

EFFECT OF INHIBITION OF S-NITROSOGLUTATHIONE  
REDUCTASE ON THE NF- $\kappa$ B PATHWAY

Sharry L. Fears

Submitted to the faculty of the University Graduate School  
in partial fulfillment of the requirements  
for the degree  
Master of Science  
in the Department of Biochemistry and Molecular Biology  
Indiana University

September 2009

Accepted by the Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Master of Science.

---

Sonal P. Sanghani, Ph.D., Chair

---

Paresh C. Sanghani, Ph.D.

Master's Thesis  
Committee

---

William F. Bosron, Ph.D.

*To my Son, Nick*

*Thank you for your encouragement, patience,  
and support.*

## ACKNOWLEDGEMENTS

I would like to thank the following people who have been a part of my life and helped me through the task of completing this master of science.

- Dr. Sonal Sanghani, for being a friend, colleague and mentor.

Thank you for believing in me and encouraging me when I would start doubting myself. Thank you for sharing your Indian culture and customs.

- Dr. Paresh Sanghani, for being a great mentor, colleague and friend.

Thank you for sharing your expertise in expressing proteins, excitement for your work, and your patience.

- Dr. William Bosron, for being a mentor, friend and colleague.

Thank you for your guidance and sharing your incredible knowledge of science.

- Lanmin Zhai, my lunch time friend and colleague. Thank you for sharing your culture and Chinese food at lunch. Thank you for all the laughter and support.

- Wil Davis, lab mate and friend. Thank you for all your expertise and guidance. Thank you for sharing your Dutch culture.

- Scheri Green, lunch time friend, thank you for being a wonderful friend and colleague. Thank you for sharing your culture with me and encouraging me to visit the Caribbean.
- Dr. Marissa Schiel, thank you for all the encouragements and for being my ‘psychiatrist’. Thank you for being a great friend and colleague.
- Darlene Lambert, for being a great friend and colleague. Thank you for all the rushed orders and late orders. Thank you for all the great times.
- Jack Arthur, for being a great friend and colleague. Thank you for always coming to my rescue with my computer issues.
- Dr. Ross Cocklin, for being a friend and colleague. Thank you for answering my many questions on yeast and mass spectrophotometry.
- Josh Heyen, for being a friend and colleague. Thank you for the interesting lunch time conversations. Thank you for being my farm cohort.

## TABLE OF CONTENTS

List of Tables .....	viii
List of Figures.....	ix
List of Abbreviations .....	x

### INTRODUCTION

I. Characterization of S-nitrosoglutathione Reductase .....	1
II. Effects of the Inhibition of GSNOR.....	6
III. Effects on NF- $\kappa$ B Pathway .....	7
IV. Small Molecule Inhibitors of GSNOR .....	10
V. Biotechnology.....	15

### MATERIALS AND METHODS

I. Cytotoxicity of GSNOR Inhibitors.....	16
II. Identification of S-nitrosylated Proteins.....	17
III. Effect of Inhibitor C3 on NF- $\kappa$ B Pathway Proteins .....	23
IV. Proteins Affected by Inhibition of NF- $\kappa$ B Pathway .....	24

### RESULTS

I. Cytotoxicity of GSNOR Inhibitors.....	26
II. Identification of S-nitrosylated Proteins.....	31

III. Effect of Inhibitor C3 on IKK $\beta$ Activity .....	36
IV. Proteins Affected by Inhibited NF- $\kappa$ B Pathway.....	42
DISCUSSION	
I. Inhibitors of GSNOR.....	44
II. Biotechnology.....	49
CONCLUSION .....	52
REFERENCES .....	53
CURRICULUM VITAE	

## LIST OF TABLES

1. <i>In vitro</i> Percent Inhibition and IC <sub>50</sub> of C1, C2, and C3.....	11
2. Compound Inhibition Comparison of ADHs .....	12
3. Treatments of RAW 264.7 cells with C3 .....	19



## LIST OF FIGURES

1. GSNOR Catalytic Reaction.....	1
2. Substrates for GSNOR .....	3
3. Structure of GSNOR Homodimer .....	4
4. GSNOR Reduction of GSNO.....	6
5. NF- $\kappa$ B Pathway .....	9
6. Inhibitors of GSNOR.....	14
7. Inhibition of A549 cells by C2 using BrdU Incorporation.....	27
8. Inhibition of A549 cells by C3 using BrdU Incorporation.....	28
9. Inhibition of A549 cells by C3 with and without TNF $\alpha$ .....	29
10. Cell Viability after 10 hours of Incubation with Inhibitors.....	30
11. Schematics of the Biotin Switch Assay.....	32
12. Nitrosylated Proteins after Treatment with C2 or C3 .....	34
13. S-nitrosylated IKK $\beta$ of Treated Samples Following Inhibition with C3 .	36
14. Effect of C3 on the Phosphorylation of I $\kappa$ B.....	38
15. Effect of C3 on the Phosphorylation of I $\kappa$ B in the Presence of a Proteasome Inhibitor .....	40
16. IKK $\beta$ Phosphorylation in A549 cells .....	41
17. Expression of ICAM-1 is Regulated by NF- $\kappa$ B .....	43
18. NO Bioactivity and Signaling Pathway.....	45
19. NF- $\kappa$ B Pathway .....	4

## LIST OF ABBREVIATIONS

12-HDDA	12-hydroxydodecanoic acid
12-ODDA	12-oxododecanoic
A549	human lung carcinoma epithelial cell line
ADH	Alcohol dehydrogenase
ADH1B	Alcohol dehydrogenase 1B; $\beta_2\beta_2$ -ADH
ADH4	Alcohol dehydrogenase 4; $\pi$ -ADH
ADH7	Alcohol dehydrogenase 7; $\sigma\sigma$ -ADH
ALF	airway lining fluid
Biotin-HPDP	N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide
BOG	$\beta$ -octyl glucoside
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
C1	3-[1-(4-acetylphenyl)-5-phenyl-1H-pyrrol-2-yl]propanoic acid
C2	5-chloro-3-{2-[(4-ethoxyphenol)(ethyl)amino]-2-oxoethyl}-1H-indole-2-carboxylic acid
C3	4-{[2-[(2-cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl]methyl} benzoic acid
DMSO	dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FDH	Formaldehyde dehydrogenase

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione reductase
GSSG	glutathione disulfide
HEN	Hepes, EDTA, neocupronine
HMGSH	hydroxymethylglutathione
HRP	horseradish peroxidase
IC <sub>50</sub>	half maximal inhibitory concentration
ICAM	intercellular adhesion molecule
IκB	inhibitor kappa B
IKKβ	inhibitor kappa B kinase beta
iNOS	Inductible nitric oxide synthase
L-NAME	N <sup>ω</sup> -Nitro-L-arginine methyl ester hydrochloride
MG-132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
mICAM	transmembrane intercellular adhesion molecule
MMTS	S-methylmethanethiosulfonate
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
NF-κB	Nuclear factor-kappa B

NO	nitric oxide
PBS	phosphate buffered saline
pI $\kappa$ B	phosphorylated inhibitor kappa B
pIKK $\beta$	phosphorylated inhibitor kappa B kinase beta
PVDF	Polyvinylidene difluoride
RAW 264.7	mouse Abelson murine leukemia virus transformed macrophage cells
RBC	red blood cells
SDS	sodium dodecyl sulfate
sICAM	soluble intercellular adhesion molecule
SNO	S-nitrosothiol
TBS	Tris buffered saline
TBS-T	Tris buffered saline with tween
TNF $\alpha$	tumor necrosis factor alpha
WT	wild type
$\beta$ ME	beta mercaptoethanol

## INTRODUCTION

### I. Characterization of S-nitrosogluthathione Reductase

S-nitrosogluthathione reductase (GSNOR) also known as glutathione-dependent formaldehyde dehydrogenase (FDH), is a zinc-dependent dehydrogenase. It is a member of the alcohol dehydrogenase (ADH) family and is also called Class III alcohol dehydrogenase. The substrate specificity of GSNOR has been well studied (Holmquist and Vallee, 1991; Wagner *et al.*, 1984). It oxidizes long chain alcohols to an aldehyde with the help of a molecule of  $\text{NAD}^+$  (Figure 1). Alcohols with a carbon chain longer than four carbons and containing a carboxyl group at the opposite end are metabolized by GSNOR much more efficiently than ethanol (Sanghani *et al.*, 2000; Wagner *et al.*, 1984) an example is 12-hydroxydodecanoic acid (Figure 2A). As the carbon chain length of alcohol increases, the  $K_m$  of the alcohol decreases (Wagner *et al.*, 1984).

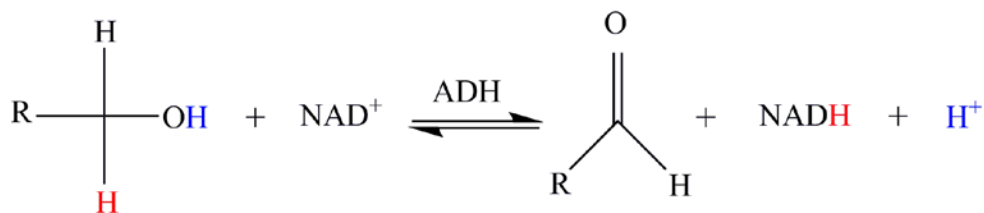
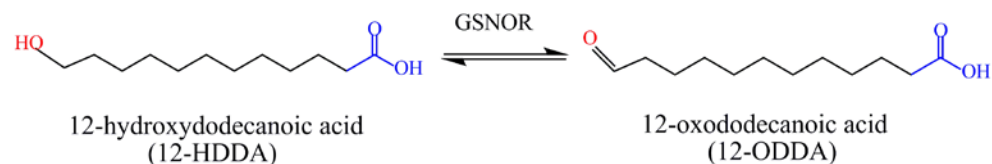


Figure 1. GSNOR Catalytic Reaction. GSNOR oxidizes an alcohol to aldehyde using  $\text{NAD}^+$  as a coenzyme.

GSNOR was initially identified as FDH because of its role in the formaldehyde detoxification pathway. Hydroxymethylglutathione (HMGS<sub>H</sub>) is formed by the spontaneous reaction of formaldehyde and glutathione (GS<sub>H</sub>). FDH oxidizes HMGS<sub>H</sub> to S-formylglutathione (Figure 2B) with the help of NAD<sup>+</sup>. S-formylglutathione is further converted to formic acid and glutathione enzymatically by S-formylglutathione hydrolase. Removal of formaldehyde from the cells protects the cells from its detrimental effects. Formaldehyde is detrimental to cells because it can modify proteins, damage membranes, and cause mutagenesis through DNA-protein cross-links (Barber and Donohue, 1998). Formaldehyde was commonly used as a fixative for cell and tissue cultures until it was declared a carcinogen.

A)



B)

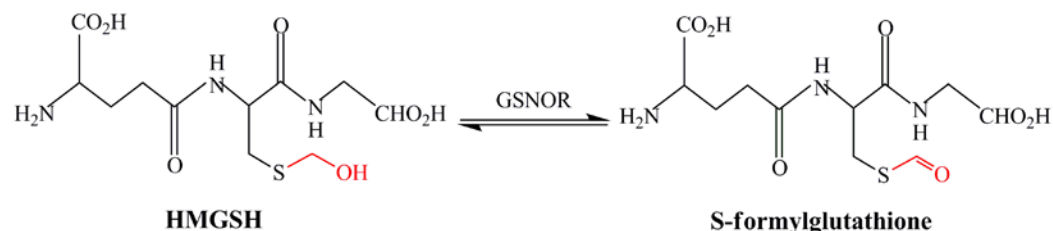


Figure 2. Substrates for GSNOR. A) *In vitro* reaction of GSNOR: 12-HDDA is an example of a long chain primary alcohol with a carboxyl group which GSNOR oxidizes to an aldehyde. B) GSNOR plays a key role in the removal of formaldehyde from the body. Formaldehyde spontaneously reacts with GSH to form HMGS, GSNOR catalyzes the reaction of HMGS to S-formylglutathione which is hydrolyzed to glutathione and formic acid by S-formylglutathione hydrolase.

The crystal structure of GSNOR has been determined (Yang *et al.*, 1997)

and the enzyme exists as a homodimer in its native state. GSNOR contains a coenzyme binding site for  $\text{NAD}^+/\text{NADH}$  and a substrate binding site for GSNO or long chain alcohols (Figure 3).

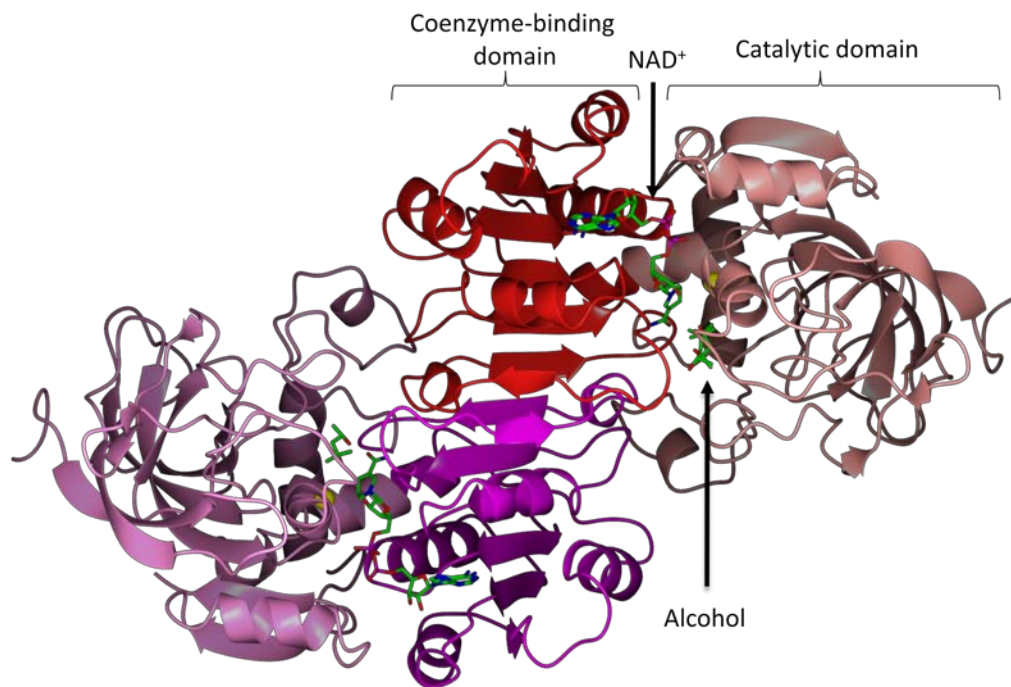


Figure 3. Structure of GSNOR Homodimer. Each GSNOR monomer contains a zinc ion, coenzyme binding site for NADH, and catalysis binding site for substrate.

GSNOR is ubiquitously expressed in tissues as compared to other ADHs (Kaiser *et al.*, 1989; Sanghani *et al.*, 2000; Estonius *et al.*, 1996). One characteristic of ubiquitously expressed genes is the absence of a TATA box or a CAAT box in its promoter region (Hur and Edenberg, 1992), as is the case in GSNOR. GSNOR is a highly conserved enzyme and can be found in both prokaryotic and eukaryotic organisms. GSNOR has been maintained throughout evolution and is vital for NO homeostasis as a regulator for protein S-nitrosation through the reduction of GSNO (Liu *et al.*, 2001). Although GSNOR is expressed



in all tissue, its activity levels are highest in the liver followed by kidney, heart, lung, spleen and thymus (Liu *et al.*, 2004).

GSNOR was very well characterized as FDH until a report by Liu *et al.* identified NADH dependent GSNO-metabolizing enzyme as FDH by mass spectrophotometry (Liu *et al.*, 2001). Subsequent studies revealed the GSNOR's oxidation rate for HMGSN is two to eight fold higher when GSNO is available than for HMGSN alone (Staab *et al.*, 2008). The only S-nitrosothiol (SNO) substrate recognized by GSNOR is GSNO (Liu *et al.*, 2004). A transnitrosation reaction transfers NO from nitrosylated proteins or S-nitrosothiols (RSNO) to glutathione to form S-nitrosoglutathione. This GSNO is finally converted to glutathione disulfide (GSSG) by a two step mechanism. First, GSNOR reduces GSNO to N-hydroxysulfenamide-glutathione (Fukuto *et al.*, 2005) in the presence of NADH followed by non-enzymatic reaction of N-hydroxysulfenamide-glutathione and glutathione to glutathione disulfide and hydroxylamine (Figure 4). Cellular GSNO is a nitric oxide reservoir that can either transfer to or remove from the proteins a NO group. Reduction of GSNO by GSNOR depletes this reservoir and therefore indirectly regulates protein nitrosylation.

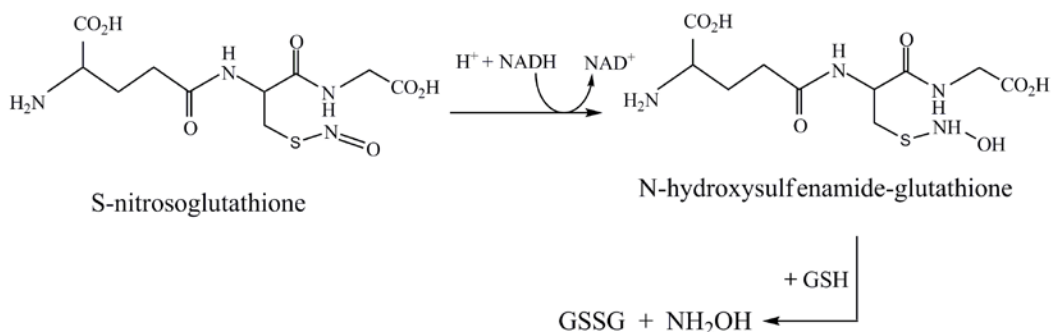


Figure 4. GSNOR Reduction of GSNO. The figure above shows the reduction of GSNO by GSNOR to a final product of GSSG and hydroxylamine.

## II. Effects of the Inhibition of GSNOR

The role of GSNOR in NO metabolism is very well established by studies in GSNOR knockout mice (Liu *et al.*, 2004). The effect of inhibition in GSNOR should result in an increase in RSNOs which is what was observed in GSNOR knockout mice (Liu *et al.*, 2004). After treatment with lipopolysaccharide, RSNOs in GSNOR<sup>-/-</sup> mice increased 3.3-fold and 29-fold over wild-type (WT) mice at 24 and 48 hours, respectively. Of the RSNOs in the GSNOR<sup>-/-</sup> mice 90% are RSNOs with molecular weights >5000 (Liu *et al.*, 2004). This increase in RSNOs effects vascular homeostasis, asthma, and cystic fibrosis. Hypotension was amplified in anesthetized GSNOR<sup>-/-</sup> mice over WT mice. The basal levels of SNOs in RBC's from GSNOR<sup>-/-</sup> mice were 2-fold higher, this amount would cause vasodilation in bioassays (Liu *et al.*, 2004). In the study by Que *et al.*, WT mice exposed to the allergen ovalbumin exhibited airway hyperresponsivity and were depleted of lung SNOs likely due to increased GSNOR activity seen in these mice.

In the same study, GSNOR<sup>-/-</sup> mice do not show airway hyperresponsivity upon exposure to ovalbumin (Que *et al.*, 2005). Using a Human Airway Bioassay technique, Gaston *et al.* documented GSNO concentrations in asthma patients to be much lower than in control patients and correlated inversely to NO concentrations (Gaston *et al.*, 1993). Elevated NO in patient's exhaled air, is one of the top symptoms in the diagnosis of asthma (Que *et al.*, 2005). A depletion of GSNO in the airway lining fluid (ALF) of asthmatic patients also correlates with this symptom. In a clinical study, asthma patients have shown to have two times higher GSNOR activity than controls and depleted GSNO and SNOs in bronchoalveolar samples (Que *et al.*, 2009). Asthma and cystic fibrosis patients have a decrease in GSNO concentration in the airway lining fluid. One treatment being investigated for cystic fibrosis patients is inhaling aerosolized GSNO (Foster *et al.*, 2003; Zaman *et al.*, 2006). GSNOR inhibitors which can increase the basal GSNO levels will be another potential therapy.

### **III. Effects on NF- $\kappa$ B Pathway**

The NF- $\kappa$ B Pathway is regulated by series of positive and negative regulatory elements. Positive regulation causes phosphorylation of IKK $\beta$ , which in turn phosphorylates I $\kappa$ B. I $\kappa$ B $\alpha$  is an inhibitory molecule that sequesters NF- $\kappa$ B in the cytosol. Phosphorylation of I $\kappa$ B $\alpha$  targets it for ubiquitination and proteasomal degradation releasing NF- $\kappa$ B (Figure 5). NF- $\kappa$ B then travels to the nucleus and initiates transcription of cytokines, chemokines, which includes genes

such as NOS, tumor necrosis factor alpha, (TNF $\alpha$ ), intercellular adhesion molecule (ICAM), and self regulation. ICAM is a transmembrane protein (mICAM) or a soluble protein (sICAM) which is produced in epithelial cells and leukocytes (Hayden *et al.*, 2006; Whiteman and Spiteri, 2008). ICAM adheres the leukocytes to the affected endothelial cells. Leukocytes are the defense mechanism of the body and will migrate into the infected tissue (Hayden *et al.*, 2006). Mice that have inadequate amounts of p65 NF- $\kappa$ B lack the ability to adhere leukocytes to the epithelial cells which slows the immune response (Hayden *et al.*, 2006).

The role of nitric oxide in regulation of NF- $\kappa$ B pathway is reviewed by Bove and van der Vliet (Bove and van der Vliet, 2006). IKK $\beta$  has been shown to be a direct target for SNO modification resulting in decreased IKK $\beta$  activity causing inhibition of NF- $\kappa$ B dependent transcription (Reynaert *et al.*, 2004). Asthma is the overstimulated inflammatory pathway in response to allergens entering the respiratory system. The fact that asthma patients have decreased amounts of GSNO could result from overproduction of GSNOR causing an imbalance of NO and S-nitrosylated proteins; therefore activating the NF- $\kappa$ B pathway and consequently the immune response. Inhibiting GSNOR would prevent the rapid removal of NO from S-nitrosylated proteins including IKK $\beta$  and therefore could impede the NF- $\kappa$ B pathway, slowing the immune response in asthma patients (Figure 5).

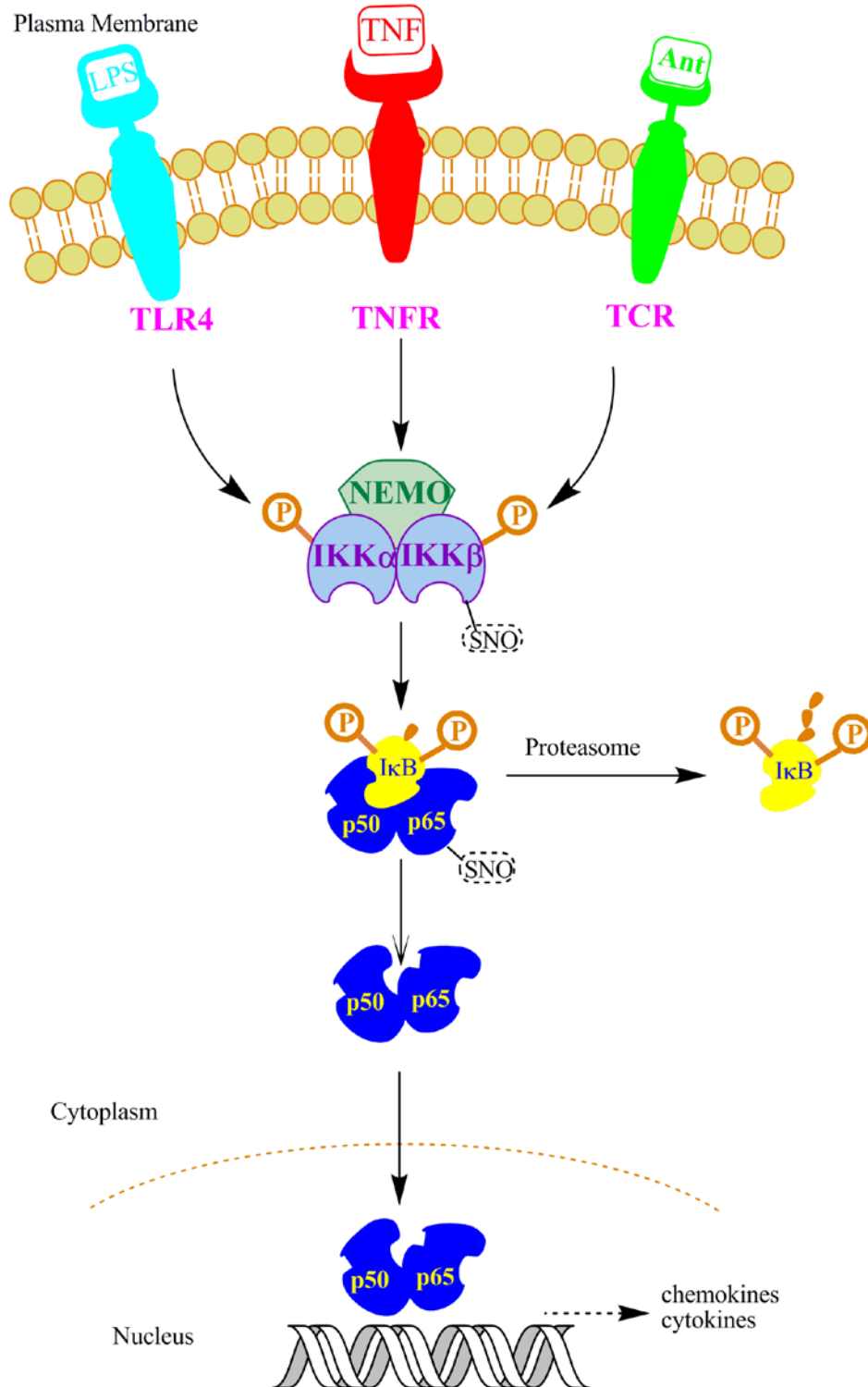


Figure 5. NF-κB Pathway. The NF-κB is a key transcription factor for the transcription of cytokines and chemokines.

#### IV. Small Molecule Inhibitors of GSNOR

High-throughput screening was performed using ChemDiv Inc's small molecule library of 60,000 compounds for inhibition of GSNOR activity in Chemical Genomics Core facility at Indiana University. Recombinant GSNOR was expressed in *E. coli* and purified using a previously described method (Sanghani *et al.*, 2000). In high-throughput screening GSNOR activity was determined using 384 well plates with substrate octanol and cofactor NAD<sup>+</sup>. The production rate of NADH absorbance at 340 nm was monitored. Potential compounds were selected based on the ability to inhibit the activity of GSNOR and retested in the laboratory using an IC<sub>50</sub> *in vitro* assay. If the compound showed a 100 fold lower IC<sub>50</sub> than the known inhibitor dodecanoic acid concentration in the IC<sub>50</sub> *in vitro* test (Table 1) they were selected for further studies. The small molecules were then tested for the inhibition of other ADHs, allowing selection of compounds that exclusively inhibit GSNOR (Table 2).

Compound Number	% inhibition		IC <sub>50</sub> at pH 7.5 μM
	pH 10	pH 7.5	
Dodecanoic acid	4	19	212
C1	78	93	1.3
C2	55	91	2.4
C3	75	95	1.1

Table 1. *In vitro* Percent Inhibition and IC<sub>50</sub> of C1, C2, C3. The percent inhibition was determined using 2 conditions. Inhibition studies at pH 10 were performed in 0.1 M sodium glycine (pH 10), 1 mM octanol, 1 mM NAD<sup>+</sup>, 0.1 mM EDTA and 50 μM inhibitor. Inhibition studies at pH 7.5 were performed in 50 mM potassium phosphate pH 7.5 containing 15 μM NADH, 10 μM GSNO, 0.1 mM EDTA and 50 μM inhibitor.

Enzyme	% Inhibition		
	C1	C2	C3
GSNOR	77	71	73
ADH1B	5	0	5
ADH7	4	13	8
ADH4	6	1	3

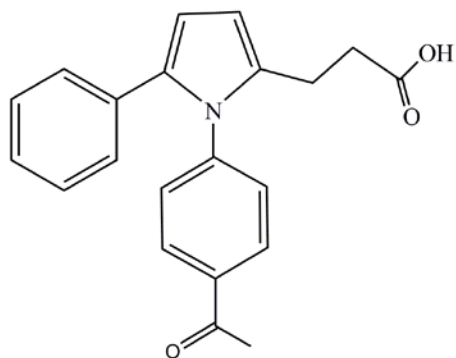
Table 2. Compound Inhibition Comparison of ADHs. Inhibition studies were performed in presence or absence of 5  $\mu$ M inhibitor at 25°C in 50 mM potassium phosphate pH 7.5 containing 0.1 mM EDTA. The enzyme activity was measured by following the changes in absorbance at 340 nm. The values show the percent reduction in the enzyme activity caused by the inhibitor. The standard errors are below 15% of the averages shown, except when the inhibition was below 20%. Studies with ADH1B ( $\beta_2\beta_2$ -ADH), ADH7( $\sigma\sigma$ -ADH), ADH4 ( $\pi$ - ADH) were performed in 0.05 % DMSO. Studies with GSNOR were performed in presence of 1 % DMSO.

From the *in vitro* IC<sub>50</sub> and inhibition studies, three candidates, C1, C2, and C3, (Figure 6A-C) were selected and assessed *ex vivo* using RAW 264.7 macrophage cells and A549 human carcinoma lung epithelial cells (Sanghani *et al.*, 2009). Of these three inhibitors all experiments were completed using C3. Inhibitor C2 experiments were performed during cytotoxicity, nitrosylation, and C1 was only analyzed early in the studies, *ie.* cytotoxicity experiments. Initially C3 showed higher level of protein nitrosylation in cells making C3 our first choice. Some experiments with C2 were included for comparison to C3.



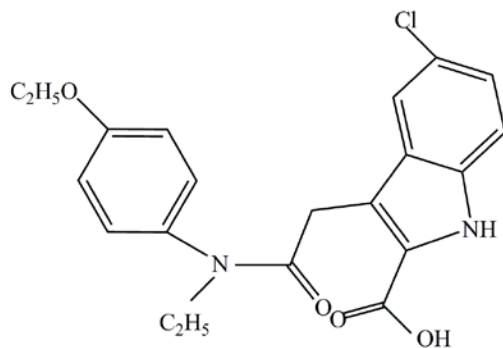
Very little data for C1 was gathered because the results did not show sufficient nitrosylation in cell lysates compared to C2 and C3. During ongoing experiments C3 seemed to exhibit more results, *ie.* detection of more nitrosylation in cell lysates, more defined IκB experiments than C2 and C3.

C1



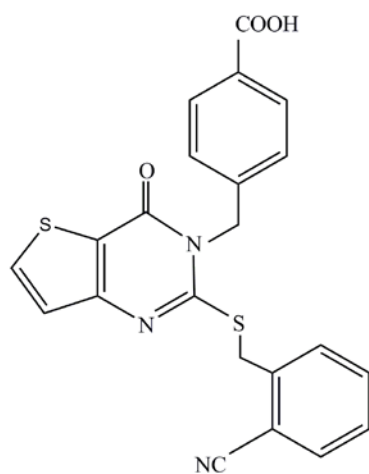
3-[1-(4-acetylphenyl)-5-phenyl-1H-pyrrol-2-yl]propanoic acid

C2



5-chloro-3-{2-[(4-ethoxyphenyl)(ethyl)amino]-2-oxoethyl}-1H-indole-2-carboxylic acid

C3



4-{{2-[(2-cyanobenzyl)thio]-4-oxothieno[3,2-d]pyrimidin-3(4H)-yl}methyl}benzoic acid

Figure 6. Inhibitors of GSNOR. Structures of the GSNOR inhibitors C1; C2; C3.

## **V. Biotechnology**

Several biotechniques were utilized to obtain the data demonstrating the effects of inhibition of GSNOR on the NF- $\kappa$ B Pathway. Western blot is a critical technique and can be used for protein identification and semi-quantitation.

Therefore selection and dilution of antibody are essential for cell culture techniques such as sterility, plating and selection of cells, are also crucial. ELISA and spectrophotometry were also utilized.

## MATERIALS AND METHODS

### I. Cytotoxicity of GSNOR Inhibitors

#### *Cell Viability*

Cell viability was determined using Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega One Assay). The Promega One Assay is a colormetric method determining viable cells by production of formazan which absorbs at 490 nm. The production of formazan is directly proportional to the number of live cells. Human lung adenocarcinoma epithelial cell line, A549 (ATCC) was cultured in ATCC F-12K media containing 10% heat inactivated FBS and 1% pen/strep, at 37°C and in 5% CO<sub>2</sub>. On day 1, 1500 A549 cells were plated in 100 µl medium per well of a 96-well tissue culture plate. The next day cells were treated with 100 µl of inhibitor C3 diluted in medium, for a final concentration 0, 10, 30, 50, 80, 110, 150 µM and incubated for 4 hours at 37°C. TNFα was added to the cells to a final concentration of 10 ng/ml and incubated for 5 days. The medium was replaced with 120 µl of fresh media containing 20 µl of the Promega One Assay Reagent. The cells were incubated again at 37 °C for 1 hour and then the absorbance was determined at 490 nm using a Molecular Devices SpectraMax 190 microplate reader.

A second experiment was completed with minor changes to determine the cell viability for inhibitors C1, C2, C3. The total time of treatment for this

experiment was 10 hours. On day 1, 1500 A549 cells were plated in 100  $\mu$ l medium per well of a 96-well tissue culture plate. The next day cells were treated with 100  $\mu$ l of inhibitor, C1, C2, or C3 diluted in medium, for a final concentration of 0, 10, 30, or 75  $\mu$ M and incubated for 4 hours at 37°C. TNF $\alpha$  was added to the cells to a final concentration of 10 ng/ml and incubated for an additional 6 hours at 37°C. Measurements were made using Promega One Assay reagent as described above.

#### *Cell Proliferation*

The cell proliferation was determined using Bromodeoxyuridine Cell Proliferation Assay kit (Calbiochem). This assay uses Bromodeoxyuridine (BrdU) as a nucleoside to replace deoxythymidine in newly synthesized DNA produced in the S-phase of the cell cycle. A549 cells were plated in 96-well plates at 1500 or 4000 cells per well in 100  $\mu$ l media and incubated overnight. The cells were treated with 0, 0.3, 1, 3, 10, 30, 50, or 100  $\mu$ M of C2 or C3 and incubated for 24 or 48 hours at 37°C. The relative amount of BrdU incorporated is then determined by an ELISA test using anti-BrdU monoclonal antibody and a secondary antibody conjugated with HRP. The HRP product is proportional to the incorporated BrdU.

## **II. Identification of S-nitrosylated Proteins**

On day 1, twelve 100 mm dishes were plated with  $3 \times 10^6$  Raw 264.7 cells per dish in 10 ml of DMEM F-12 media (ATCC) + 10% heat inactivated fetal

bovine serum (Atlanta Biologicals) + 1% penicillin/streptomycin (Invitrogen). On day 2, the medium in the dishes was replaced with 9 ml of fresh DMEM medium prior to treatment. Cells were treated according to Table 3 to obtain a final concentration of, 33  $\mu$ M for C3 and 1.1  $\mu$ M for L-NAME. Cells were incubated at 37°C and 5% CO<sub>2</sub> with these compounds for 2, 4, 8 or 24 hours then lysed with HEN lysis buffer (250 mM HEPES pH 7.7, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM Neocupronine, 1% Nonidet P-40 (Sigma) and 10 mM S-methyl methanethiosulfonate (MMTS)). Because of the photosensitivity of the SNOs', all handling of samples after treatment was completed under special yellow lights.

Plate #	Treatment	Time
1, 7	DMSO	4hr
2, 8	C3 33 $\mu$ M	2 hr
3, 9	C3 33 $\mu$ M	4hr
4, 10	C3 33 $\mu$ M + 1.1 $\mu$ M L-NAME simultaneous treatment	4hr
5, 11	C3 33 $\mu$ M	8 hr
6, 12	C3 33 $\mu$ M	24 hr

Table 3. Treatments of Raw 264.7 cells with C3.

#### *Lysate Preparation*

After treatment, in controlled lighting, the medium was removed and the cells were washed twice with 10 ml of phosphate buffer saline (PBS). After removal of the PBS, cells were scraped off in 500  $\mu$ l of freshly made HEN lysis buffer. Lysate was placed in a microfuge tube and vortexed. Samples were then centrifuged at 16,100 x g for 10 minutes at room temperature. The supernatant (lysate) was removed, snap frozen using liquid nitrogen and stored at  $-20^{\circ}\text{C}$ .

Protein concentration of the lysates was determined by Bio-Rad Protein assay using 2.5  $\mu$ l of lysate per assay and bovine serum albumin (BSA) as standard.

### *Biotin Switch Assay*

The lysates from the samples above were analyzed for S-nitrosothiols using the Biotin Switch Assay described by Jaffrey *et al* with a few modifications (Jaffrey and Snyder, 2001; Wang *et al.*, 2008; Zhang *et al.*, 2005). The product of this assay switches the S-nitrosothiols with biotin. To 200 µg of total protein from above lysates was added, 80 µl of 25% sodium dodecyl sulfate (SDS), 6 µl of 2 M MMTS (final concentration 10-15 mM MMTS) and Hen buffer to a total volume of 1 ml. Samples were incubated in a 50°C water bath for 25 minutes with occasional vortexing.

Five ml spin columns were prepared with Sephadex G25 resin equilibrated in chelexed PBS containing 1% SDS and 0.1 mM EDTA and centrifuged at 1000 x g for 2 minutes to pre-pack the columns. The samples were loaded onto the columns and centrifuged at 1000 x g for 2 minutes. The eluant was sequentially passed through two additional spin columns. The volume of the final eluant was measured and divided equally into 2 microfuge tubes, labeled **A** and **B** for the Biotin-HPDP labeling. Two hundred µl of 4 mM biotin-HPDP + 250 µl of dilution buffer (100 mM HEPES pH 7.7, 2.5 mM EDTA, 0.25 mM Neocuproine) were added to tubes labeled A. Two hundred µl of 4 mM Biotin-HPDP + 48 µl of 500 mM ascorbic acid + 200 µl regular water + 25 µl saturated cuprous chloride (CuCl) were added to tubes labeled B for biotin labeling. Both sets of tubes were kept in the dark at 25°C for 3 hours. Five ml spin columns containing Sephadex G25 resin, equilibrated with 25 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA



and 0.5%  $\beta$ -octyl glucoside (BOG) were pre-packed by centrifugation at 1000 x g for 2 minutes. Samples were loaded onto the columns and centrifuged at 1000 x g for 2 minutes. The eluant was then sequentially placed on two additional columns to remove free biotin. Protein concentration was determined using 25  $\mu$ l of each sample, using BCA Protein Assay (Pierce). Five  $\mu$ g of each sample was transferred to a new tube and  $\beta$ -ME free Lamelli buffer was added in preparation for the SDS-PAGE/Western blot assay.

*Western Blot for Biotin Detection.*

The biotin labeled lysate was analyzed by Western blot to establish the nitrosylation effect of the GSNOR inhibitors over selected time points. Five  $\mu$ g of protein from samples described above was boiled for 5 minutes and centrifuged at 16100 x g for 5 minutes. One  $\mu$ g of protein was loaded on a precast 10% acrylamide Tris-HCl gel (BioRad). Gel was electrophoresed for 2 hours at 120V. The proteins were immediately transferred to a PVDF membrane using the BioRad Criterion Transfer Blotting apparatus with Tris/Glycine buffer pH 8.3 containing 10% methanol for 45 minutes at 100 V or overnight at 30V at 4°C. The blot was blocked with 5% milk in Tris buffered saline containing 0.1% Tween 20 (TBS-T). The blot was rinsed with TBS-T and probed with an anti-biotin HRP conjugated antibody (Sigma) for 1 hour at room temperature (RT). The blot was rinsed 3 x 10 minutes with TBS-T and developed using ECL Plus chemiluminescence kit (GE Healthcare).

### *Streptavidin Precipitation*

The remaining lysate of the samples which were used in the Biotin Switch assay was subjected to acetone precipitation. The samples were centrifuged and the protein pellet was dissolved in 30  $\mu$ l of 8 M guanidine and 170  $\mu$ l of 10 mM potassium phosphate ( $KP_i$ ) pH 8.0. Protein concentration was determined by the Bio-Rad Protein Assay using BSA as standard.

Twenty  $\mu$ l of streptavidin agarose resin (Pierce, Rockford, IL) equilibrated in 25 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA, and 1% BOG (neutralization buffer) were added to equal amounts of protein for all samples. The samples were diluted to 1 ml with neutralization buffer and incubated for 4 hours at 4°C with shaking. Samples/beads were centrifuged at 100 x g for 30 seconds, supernatant discarded, the beads were then washed 5 x 1 ml of washing buffer (25 mM HEPES pH 7.7, 600 mM NaCl, 1 mM EDTA, 1% BOG). The samples were incubated with Lamelli buffer containing 500 mM  $\beta$ -mercaptoethanol ( $\beta$ ME) for 2 hours at 40°C with shaking. Samples were boiled for 5 minutes, centrifuged at 9300 x g for 5 minutes, and all of the supernatant was loaded on a Precast 10% acrylamide Tris-HCl gel (Bio-Rad).

### *Western Blot of Streptavidin Precipitated Proteins*

The SDS-PAGE gel was immediately transferred to a PVDF membrane using the Criterion Transfer Blotting apparatus (Bio-Rad) with Tris/Glycine buffer pH 8.3 with 10% methanol for 45 minutes at 100 V or overnight at 30 V. The blot was blocked with 5% milk in Tris buffered saline and 0.1% Tween 20 (TBS-T).

The blot was rinsed with TBS-T and probed with the primary antibody IKK $\beta$  (Cell Signaling Technology) in 5% BSA-TBS-T overnight at 4°C. The blot was rinsed 3 x 10 minutes with TBS-T and incubated with HRP conjugated secondary antibody for 1 hour at room temperature, rinsed again 3 x 10 minutes with TBS-T. Blot bound HRP was detected using ECL Plus chemiluminescence kit (GE Healthcare).

### **III. Effect of Inhibitor C3 on NF- $\kappa$ B Pathway Proteins**

#### *Cell Treatment and Lysate Preparation*

Two hundred thousand A549 cells, (ATCC) were plated in 2 ml of F-12K Media containing 10% heat inactivated FBS and 1% pen/strep into 35 mm dishes. Next day, the medium was replaced with 2 ml of fresh medium prior to treatment. Samples were incubated with inhibitor C3 at concentrations of 0, 30, 50, or 100  $\mu$ M for 4 hours at 37°C then incubated with 10 ng/ml of the cytokine, TNF $\alpha$ , (Invitrogen) for 5 minutes at 37°C. The medium was quickly removed and the cells were rinsed with cold PBS. The cells were quenched with Lamelli buffer containing 50 mM NaF then extracted by scraping the dish clean. Lysate was vortexed, centrifuged briefly, boiled for 5 minutes, centrifuged at 16,100 x g for 5 minutes. Equivalent volumes of sample supernatants were loaded onto a precast 10% polyacrylamide Tris-HCl gel. A second experiment was completed with pre-treatment of C3 at concentrations 0, 50 and 100  $\mu$ M for 4 hours. At the 3 hour mark MG-132, a known proteasome inhibitor, was added to a final concentration

of 40  $\mu$ M). Then after 4 hours total, TNF $\alpha$  was added to specific samples for 5 minutes at 10 ng/ml.

#### *Western Blot*

The gel was transferred to a PVDF membrane and the membrane was blocked with 5% milk in TBS-T. The blot was then probed overnight at 4°C with primary antibodies phospho-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , phospho-IKK $\alpha/\beta$ , and GAPDH (Cell Signaling Technology and Santa Cruz Biotechnology) in 5% BSA in TBS-T. Appropriate secondary antibodies conjugated with HRP were incubated for 1 hour at RT. HRP was detected using ECL Plus chemiluminescence kit (GE Healthcare).

### **IV. Proteins Affected by Inhibition of NF- $\kappa$ B Pathway**

#### *Cell Extract Preparation*

A549 cells were plated at 50,000 cells per well of a 24 well plate in 1 ml of F12K Medium containing 10% heat inactivated FBS and 1% pen/strep on the day before treatment. The cells were treated with 0, 10, 20, 30 and 50  $\mu$ M of C3 for 4 hours at 37°C. TNF $\alpha$  was added to the appropriate wells for a final concentration of 10 ng/ml and incubated for 6 hours at 37°C. The reaction was quenched using Lamelli buffer (no  $\beta$ ME). Cells were scraped from wells and placed in microfuge tubes. Cell extracts were frozen overnight at -20°C. Protein concentration was determined using BCA Protein Assay (Pierce). Two  $\mu$ l of  $\beta$ ME was added to each sample. Samples were vortexed, boiled for 5 minutes centrifuged at 16,100 x g for

5 minutes. Equivalent amounts of supernatant (30  $\mu$ l) were loaded onto a precast 10% acrylamide Tris-HCl gel.

#### *Western Blot*

Gel was immediately transferred as written above. The blot was probed with primary antibodies for ICAM, iNOS, COX2 and GAPDH (Santa Cruz Biotechnology) by incubating for 1-3 hours at RT. The blot was rinsed with TBS-T followed by incubation with the appropriate HRP conjugated antibody for 1 hour at RT. HRP signal on the blot was detected using ECL Plus chemiluminescence kit (GE Healthcare).

## RESULTS

### I. Cytotoxicity of GSNOR Inhibitors

Two methods were used to analyze the cytotoxicity of the inhibitors. The first method Promega One Assay, determines the number of live cells by detecting the amount of NADPH or NADH produced and present in the cytosol of the cell. The NADH or NADPH of live cells is the electron donor which converts MTS tetrazolium to formazan. The final product, formazan, is located in the cytoplasm, plasma membranes and other cellular compartments with a minor fraction in the mitochondria (Bernas and Dobrucki, 2002). The second assay, BrdU Assay measures the cells that can replicate its DNA in the S-phase of the cell cycle. The two types of assays were completed to determine the effect of the inhibitor on the cells. Is the inhibitor detrimental to the cells in 10 hours, or over longer periods of time, 48 hours. Both assays were used to determine how the inhibitor was affecting the cell *ie.* stopping replication or causing apoptosis.

#### *Cell Proliferation*

Cell Proliferation was evaluated using the BrdU Assay to determine the cytotoxicity of the inhibitors. The  $IC_{50}$  of C2 and C3 for the inhibition of A549 cells was found to be  $60 \mu M \pm 3$  and  $20 \mu M \pm 5$  respectively (Figure 7 and 8). These data suggests that cell proliferation is interrupted during long continuous exposure to the inhibitor (48 hours). This experiment also shows inhibitor C2 is 3 fold less cytotoxic than C3.

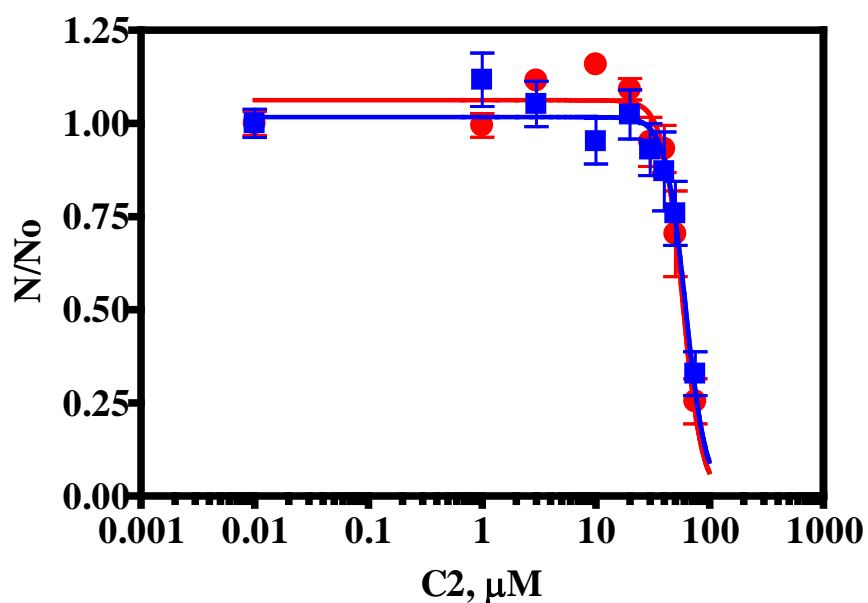


Figure 7. Inhibition of A549 cells by C2 using BrdU Incorporation. The  $\text{IC}_{50}$  of duplicate assays was analyzed using the BrdU Cell Proliferation Assay kit (Calbiochem). The  $\text{IC}_{50}$  of the inhibitory compound C2 in A549 cells after incubating with the treatment for 48 hours was  $60 \mu\text{M} + 3$  ( $n=12$ ).  $\text{IC}_{50}$  was calculated using equation  $Y = \text{Bottom} + (\text{TOP} - \text{Bottom}) / (1 + (X/\text{IC}_{50})^h)$  in GraphPad Prism 4.

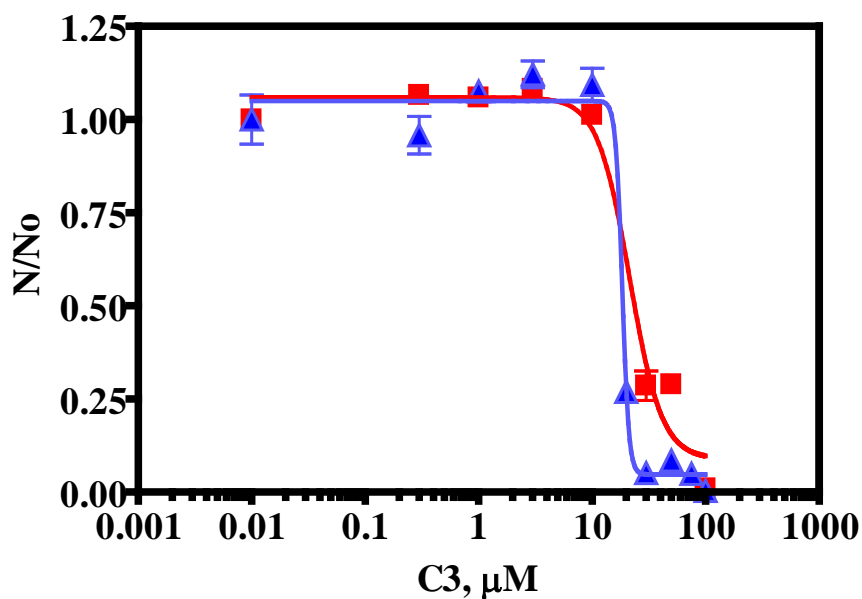


Figure 8. Inhibition of A549 cells by C3 using BrdU Incorporation. The  $\text{IC}_{50}$  of duplicate assays was analyzed using the BrdU Cell Proliferation Assay kit (Calbiochem). The  $\text{IC}_{50}$  of the inhibitor C3 was  $20 \mu\text{M} \pm 5$  ( $n=12$ ), in A549 cells after 48 hours incubation with the compound.

### *Cell Viability*

The cell viability for inhibitor C3 was also determined using the Promega One Assay after 5 days of incubation with C3 in the presence or absence of  $\text{TNF}\alpha$ . The  $\text{IC}_{50}$  of C3 without  $\text{TNF}\alpha$  was  $42 \mu\text{M} \pm 12$  and  $40 \mu\text{M} \pm 11$  with  $\text{TNF}\alpha$  (Figure 9).

The BrdU Assay of inhibitor C3 gives an  $\text{IC}_{50}$  two times lower than the Promega One Assay ( $20\mu\text{M}$  vs  $40 \mu\text{M}$ ). The difference of these results is attributed to the method of detection of cell cytotoxicity in the assays. The BrdU



Assay measures the cell division by analyzing the new DNA synthesized while Promega One Assay analyzes the NADH production of the cell. Live cells which are functional can produce NADH but may not be able to replicate.

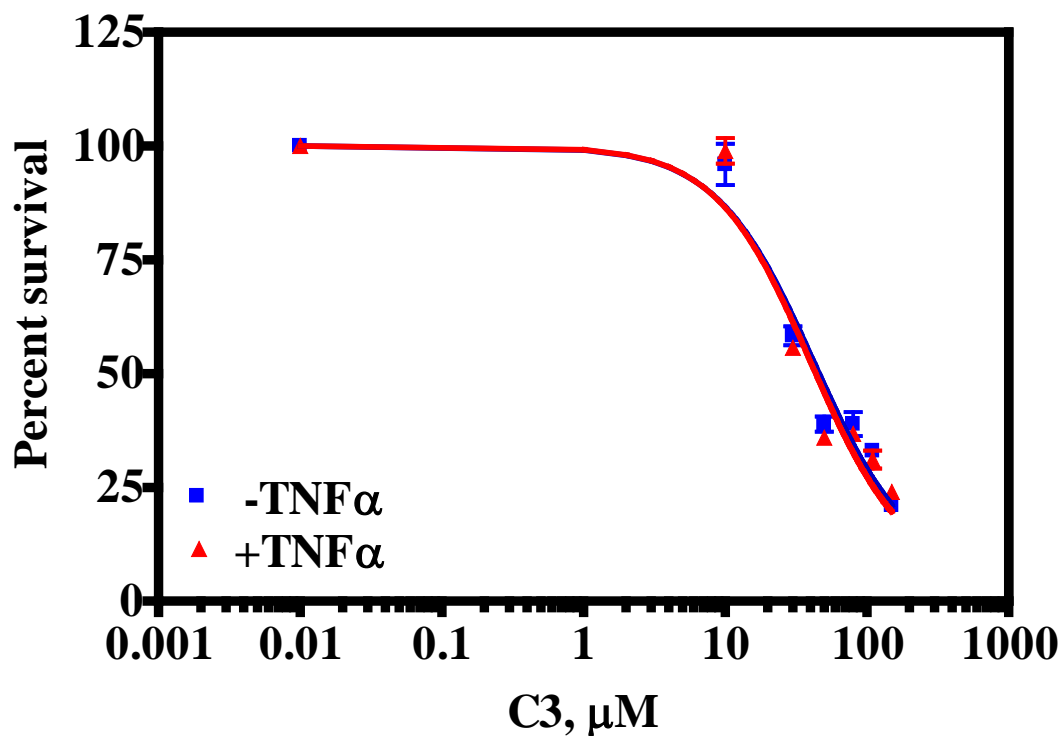


Figure 9. Inhibition of A549 cells by C3 with and without TNF $\alpha$ . The IC<sub>50</sub> was analyzed using Promega CellTiter 96 AQueous One Solution Cell Proliferation. The IC<sub>50</sub> of the inhibitor C3 was 41  $\mu\text{M}$   $\pm$  11 (n=8), in A549 cells after 5 days of incubation the compound and in the prescence (42  $\mu\text{M}$  +12) or absence (40  $\mu\text{M}$  + 11) of TNF $\alpha$ .

After determining the IC<sub>50</sub> of the compounds, a cell viability experiment was designed to establish the condition of the A549 cells treated with inhibitors C1, C2, and C3, under experimental conditions. The experimental conditions

were 4 hours of preincubation with cells and compound and then 6 additional hours with TNF $\alpha$ . Most of the data collected to study the NF- $\kappa$ B pathway was conducted within these limits. Inhibitors C1, C2, and C3 show a minimal change when normalized to untreated cells with a cell viability above 90% for the maximum inhibitor concentration tested, 75  $\mu$ M (Figure 10). This cell viability of 90% provides confidence that the cells can withstand exposure to inhibitors C1, C2 and C3 when treated with TNF $\alpha$  for short time periods. This information also provides confidence in the results of the data used to define the inhibitor C3's effect on the NF- $\kappa$ B pathway.

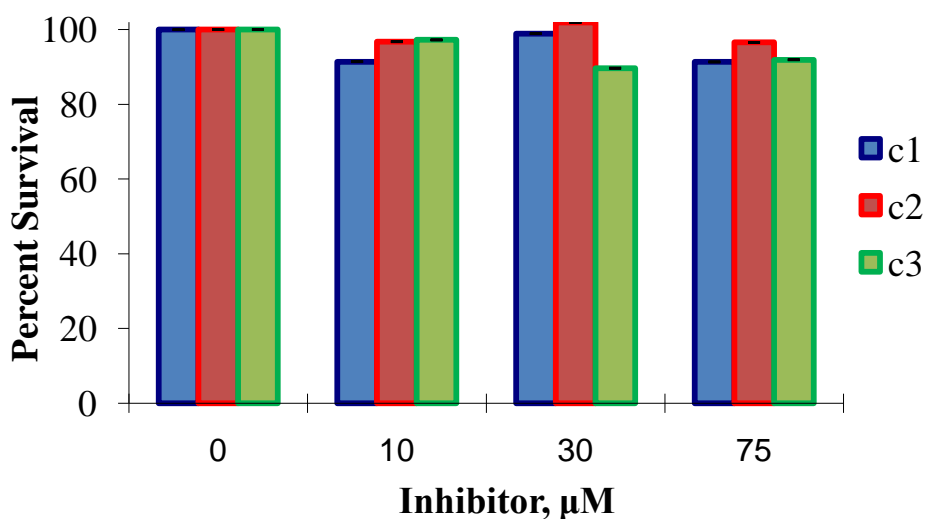


Figure 10. Cell Viability after 10 hours of Incubation with Inhibitors. A549 cells were treated with C1, C2, or C3 at indicated concentrations for 10 hours (4 hours preincubation and additional 6 hours after TNF $\alpha$  was added) and the viability was determined using the Promega CellTiter 96 AQueous One Solution Reagent.

## **II. Identification of S-nitrosylated Proteins**

The effects of GSNOR inhibition on the S-nitrosylation of cellular proteins were examined using the Biotin Switch assay. The nitrosylated proteins were labeled with biotin for analysis using the Biotin Switch Assay. The Biotin Switch Assay is necessary because S-nitrosothiol groups are very unstable and easily lost during sample treatment. This assay is a multiple step assay which removes the S-nitrosothiol group linked to the cysteines and replaces it with biotin, see Figure 11. The detection of the biotin in the biotin + ascorbate samples in the following experiments would be equivalent to the analyzing S-nitrosothiol.

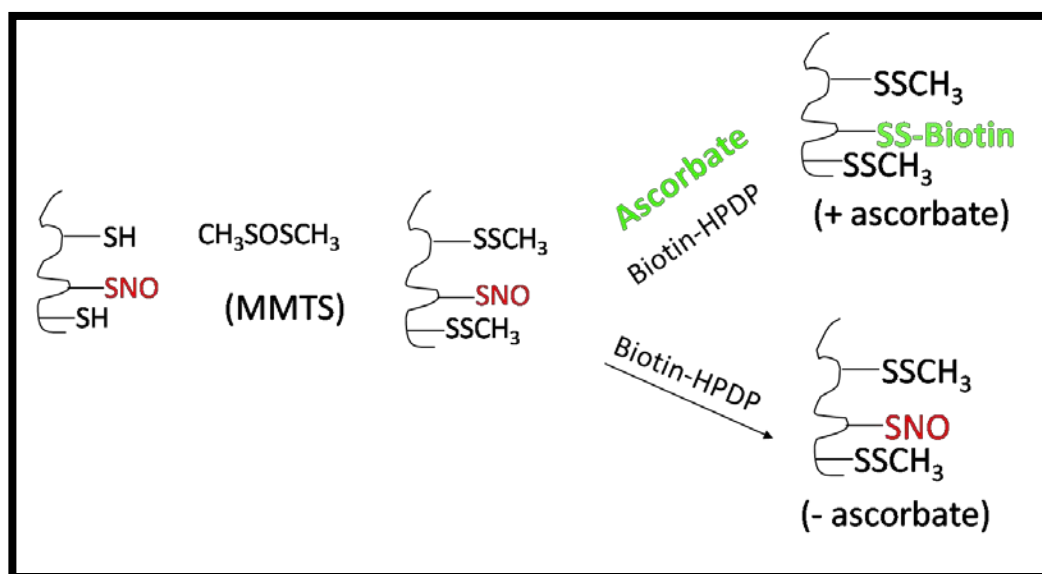
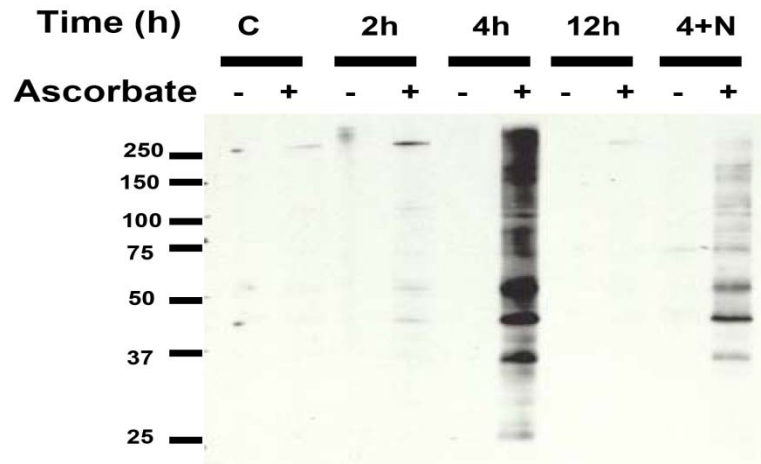


Figure 11. Schematics of the Biotin Switch Assay. Methyl methanethiosulfonate (MMTS) is first added to the lysate to block the free thiols (SH) of proteins with a methyl group. After free MMTS is removed the sample is split and N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP) plus or minus ascorbate (ascorbate, a weak reduction agent, can specifically reduce S-nitrosothiols to thiols) is added to the sample. These free thiols generated from SNO are then crosslinked with biotin using biotin-HPDP. The free biotin-HPDP is removed by gel filtration.

Raw 264.7 cells were treated with C2 or C3 for varied lengths of time, alone or in presence of nitric oxide synthase inhibitor, L-NAME (Sigma). L-NAME is an analog of arginine that inhibits the production of NO by inhibiting nitric oxide synthase. The Western blots in Figure 12 shows that nitrosylated proteins accumulate with time in cells treated with compound C2 and C3. The nitrosylation of intracellular proteins is evident following a 2 hour incubation with C3 and peaks at 8 hours before coming down to basal levels after 24 hours. Treatment with C2, nitrosylation peaked at 4 hours and back to basal levels at 12

hours. The amount of SNOs is similar in untreated cells and those treated with both C2 and L-NAME or C3 and L-NAME simultaneously. The L-NAME data illustrate the need of an active nitric oxide synthase to increase the accumulation of nitrosylated proteins and the inhibitor data suggest that inhibiting GSNOR increases the accumulation of large molecular weight SNOs in the cell.

A)



B)

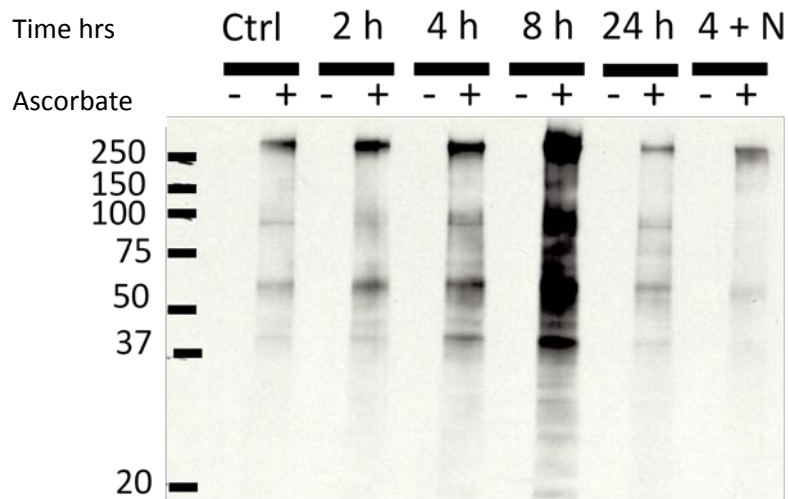


Figure 12. Nitrosylated Proteins after Treatment with C2 or C3. Cells were treated with 33  $\mu$ M C2 or C3 for varied lengths of time (0, 2, 4, 8, 12 or 24 hours) alone or in combination with 1.1 mM L-NAME for 4 hours (lane 4+N). At indicated times, the cells were quenched and the lysate was analyzed for S-nitrosothiol content by the biotin switch assay. Equal amounts of proteins were loaded in each lane and the degree of biotinylation (and hence S-nitrosylation) determined using an anti-biotin antibody. A) A549 cells treated with C2; B) RAW 264.7 cells treated with C3.

With the results of S-nitrosylated proteins increasing with time in cells treated with C2 or C3, the next step was to examine the effects of GSNOR inhibition on proteins reported to be affected by nitrosylating agents like S-nitrosoglutathione or S-nitrosocysteine. Several proteins in the NF- $\kappa$ B signaling pathway have been reported to be regulated by S-nitrosylation (Reynaert *et al.*, 2004). Inhibitor Kappa B Kinase beta (IKK $\beta$ ) was reported to be the most upstream nitrosylated protein in the NF- $\kappa$ B activation pathway. The effect of GSNOR inhibition on the S-nitrosylation of IKK $\beta$  was studied in RAW 264.7 cells using the biotin switch assay.

The biotinylated proteins were isolated using streptavidin agarose and subjected to Western blotting for the detection of specific proteins. The Western blot in Figure 13 shows IKK $\beta$  to be nitrosylated in RAW 264.7 cells following the inhibition of GSNOR with C3. The extent of S-nitrosylation of IKK $\beta$  also increased with the length of exposure of cells to C3. Concomitant treatment of cells with L-NAME and C3 did not change the S-nitrosylation level of IKK $\beta$ . This suggests the inhibition of GSNOR with C3 increases the accumulation of nitrosylated IKK $\beta$  over time but does not nitrosylate IKK $\beta$ .

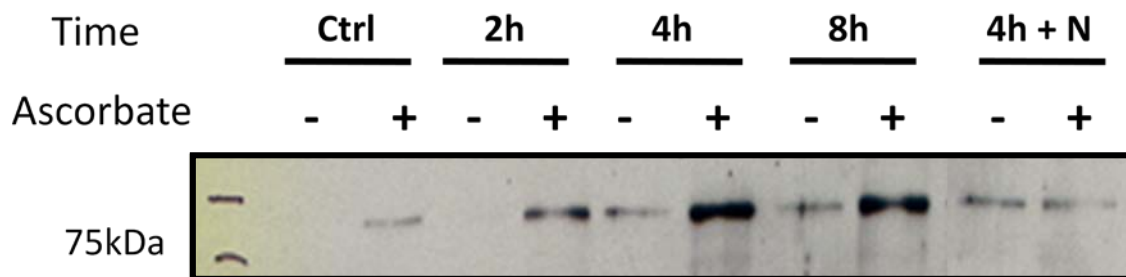


Figure 13. S-nitrosylated IKK $\beta$  of Treated Samples Following Treatment with C3. RAW 264.7 cells were treated with C3 for 0, 2, 4, 8 hours and 4 hours + L-NAME. After the S-nitrosylated proteins were labeled with Biotin, equal amounts of protein were precipitated using streptavidin agarose beads. The above Western blot was probed with anti-IKK $\beta$  antibody to demonstrate the effect of the inhibitor on the nitrosylation of protein IKK $\beta$ .

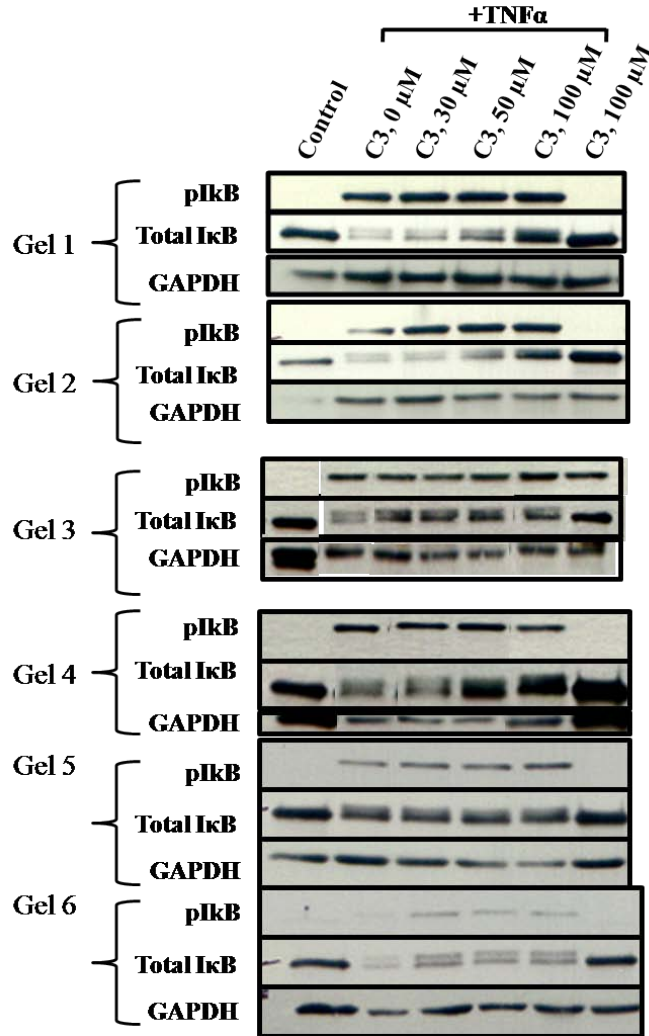
### III. Effect of Inhibitor C3 on IKK $\beta$ Activity

The inhibition of GSNOR does affect the nitrosylation of IKK $\beta$  in A549 cells, but will this nitrosylation affect the activity of IKK $\beta$ ? To determine if and how nitrosylation of IKK $\beta$  would affect its activity and signaling in the NF- $\kappa$ B pathway, cells were treated with C3 and then TNF $\alpha$ . TNF $\alpha$  stimulates the TNF receptor in the plasma membrane which initiates a cascade of kinase activity in the cytoplasm that phosphorylates IKK $\beta$  which phosphorylates the Inhibitor kappa B protein (I $\kappa$ B). This phosphorylation of I $\kappa$ B, (pI $\kappa$ B) activates the ubiquitination and proteasome degradation of I $\kappa$ B releasing and therefore allowing the translocation of NF- $\kappa$ B to the nucleus, Figure 5. If the activity of IKK $\beta$  is affected, a difference in the extent of phosphorylation of I $\kappa$ B should be detectable. The Western blot in Figure 14 demonstrates the effect of C3 on the phosphorylation of I $\kappa$ B in A549



cells. The amount of phosphorylation of I $\kappa$ B from TNF $\alpha$  stimulated cells appears to be the same as the untreated cells (Figure 14A). But after probing for I $\kappa$ B which identifies both I $\kappa$ B and pI $\kappa$ B, the blot shows a significant increase in total I $\kappa$ B with increasing concentrations of GSNOR inhibitor (n=6 \*  $p < 0.05$ , \*\*  $p < 0.01$ ) (Figure 14B). The higher amount of total I $\kappa$ B could be due to decreased IKK $\beta$  activity or decreased proteasomal degradation of I $\kappa$ B $\alpha$  preventing the release of NF- $\kappa$ B.

A)



B)

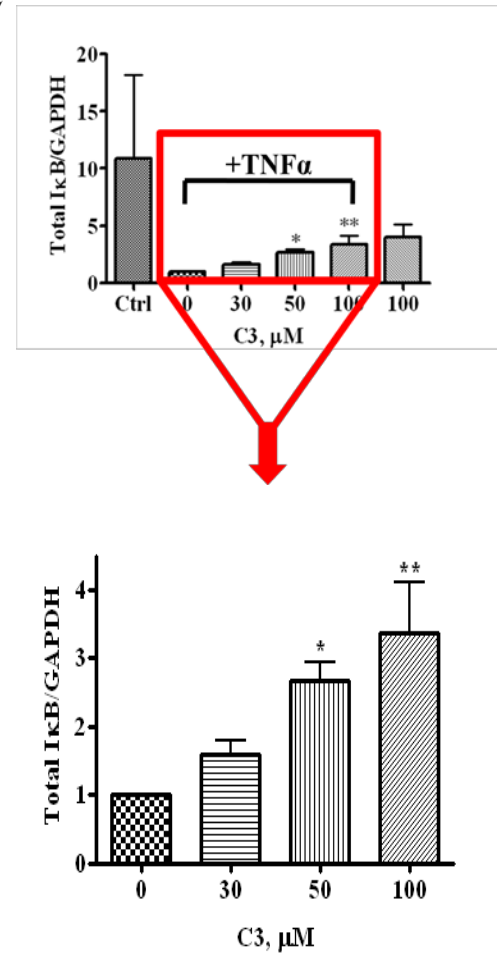


Figure 14. Effect of C3 on the Phosphorylation of IkB. A) Western Blot of A549 cells treated with C3 (0, 30, 50, 100  $\mu$ M) for 4 hours, then 10 ng/ml of TNF $\alpha$  for 5 minutes or just compound alone or control. B) Analysis of Western Blot using a densitometer (Quantity One Bio-Rad System) and a magnification of the TNF $\alpha$  treated samples, \* $p < 0.05$ , \*\* $p < 0.01$  using GraphPad Prism 4 one way anova and Tukey t-test statistics on 6 sets of data from 3 independent experiments.

The addition of proteasome inhibitor, MG-132, in the last hour of the C3 pretreatment (Figure 15) illustrates a much better account of the reaction of the cells to compound C3. The accumulation of pI $\kappa$ B in cells treated with TNF $\alpha$  and MG-132 demonstrates inhibition of proteasomal degradation of I $\kappa$ B. When cells are treated with compound C3, MG-132, and TNF $\alpha$ , the amount of pI $\kappa$ B decreases significantly with increasing concentrations of compound C3 and total I $\kappa$ B appears unchanged. In conclusion, the decreased phosphorylation of I $\kappa$ B $\alpha$  in the presence of compound is most likely due to decreased IKK $\beta$  activity and not due to decreased proteasomal activity.

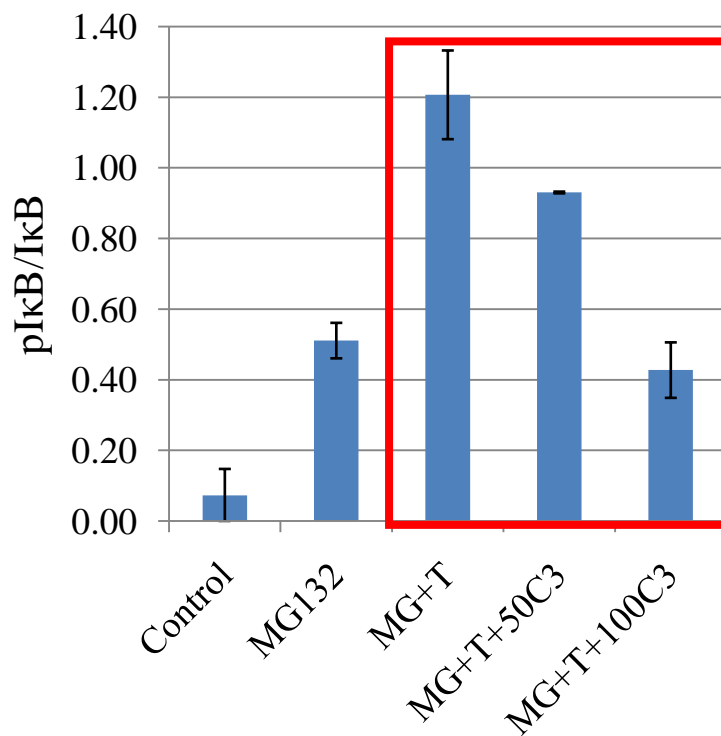
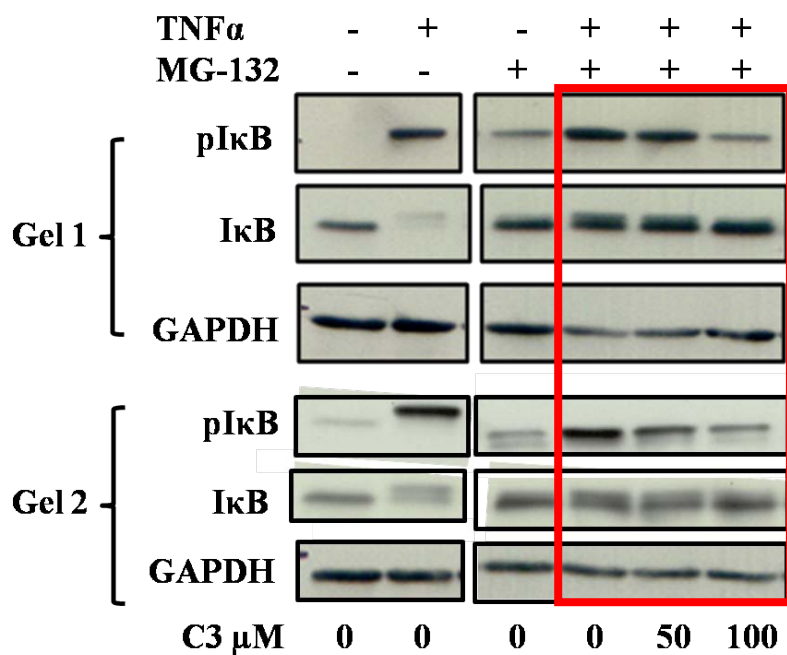


Figure 15. Effect of C3 on the Phosphorylation of I $\kappa$ B in the Presence of a Proteasome Inhibitor. A549 cells were treated with C3 as in Figure 14A plus a known proteasome inhibitor MG-132 1 hour prior to TNF $\alpha$  treatment. Graph average of 2 sets of data.

A dose-dependent increase in nitrosylation of IKK $\beta$  was shown in Figure 13. Next, we investigated whether increased nitrosylation affects phosphorylation of IKK $\beta$  or total amount of IKK $\beta$ . As shown in Figure 16 no change in phosphorylation of IKK $\beta$  and no major difference in the accumulation of IKK $\beta$  with increasing amounts of C3 was observed. This would suggest that nitrosylation of IKK $\beta$  affects its activity but not due to a decrease in phosphorylation.

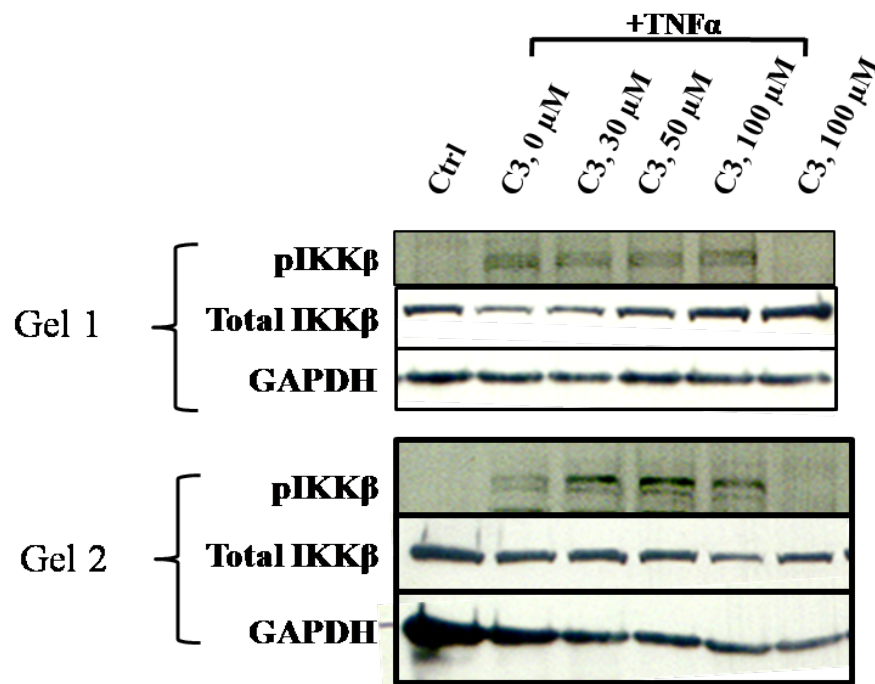


Figure 16. IKK $\beta$  Phosphorylation in A549 cells. A549 cells treated with 0, 30, 50, 100  $\mu$ M of C3 for 4 hours and 10 ng/ml of TNF $\alpha$  for 5 minutes assayed by Western blot and probed with anti-pIKK $\beta$ , anti-IKK $\beta$  and anti-GAPDH antibodies.

#### **IV. Proteins Affected by Inhibited NF- $\kappa$ B Pathway**

ICAM is one of the proteins whose expression is regulated by the transcription factor NF- $\kappa$ B. To observe the effects of this protein after inhibiting GSNOR, A549 cells were incubated with inhibitor C3 at varying concentrations and the NF- $\kappa$ B pathway was induced by TNF $\alpha$ . The amount of ICAM-1 produced decreased, with increasing concentrations of inhibitor C3 (Figure 17). This data reveals that C3 inhibits the NF- $\kappa$ B pathway.

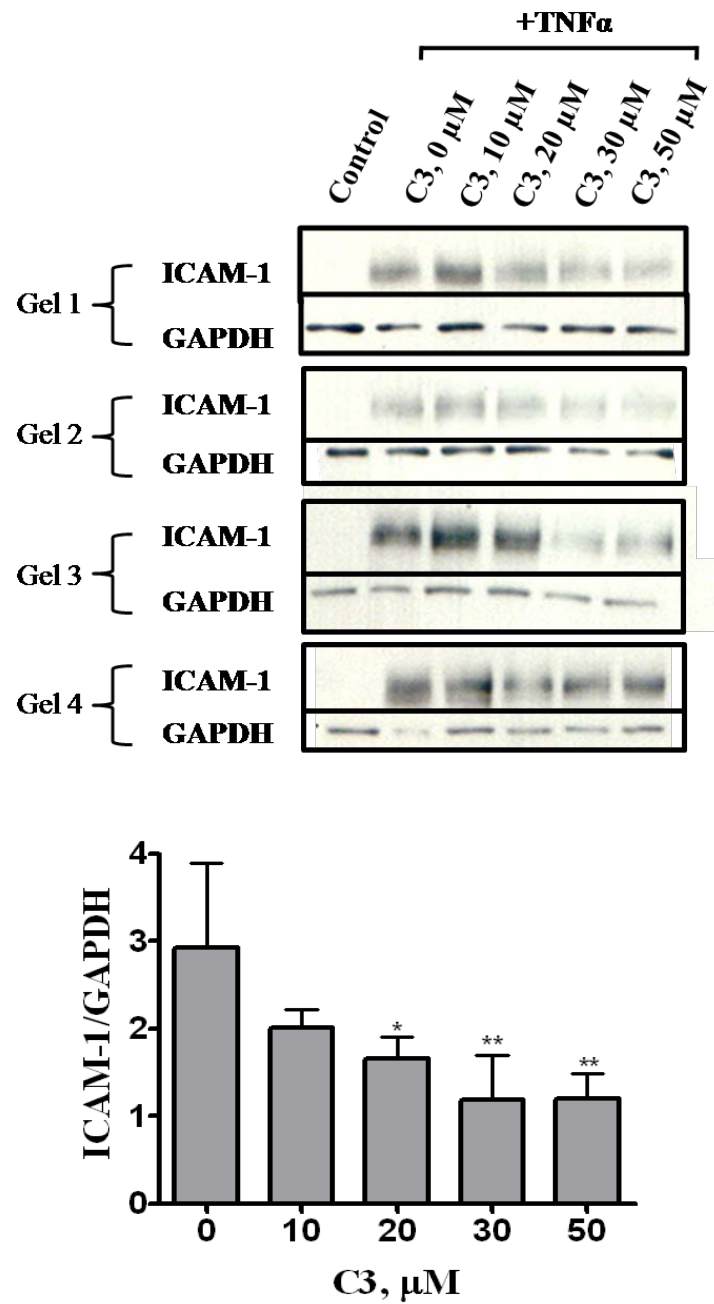


Figure 17. Expression of ICAM-1 is Regulated by NF- $\kappa$ B. Increasing the concentration of inhibitor C3, decreases the amount of ICAM-1 in the A549 cells pre-treated for 6 hours with C3 and then an additional 4 hours with TNF $\alpha$ . Statistics on 3 sets of data, \*p<0.05 and \*\*p<0.01.

## DISCUSSION

### I. Inhibitors of GSNOR

The cytotoxicity of GSNOR inhibition is not known. After 48 hours of exposure, GSNOR inhibitors C2 and C3 demonstrate a moderate cytotoxicity level on the lung epithelial carcinoma cells at low concentrations by hindering cell proliferation. Compounds inhibiting GSNOR activity could be a potential cytotoxic drug at higher concentrations. Cell viability under experimental conditions revealed live cells for the duration of the experiments. These specific inhibitors, C2 and C3 present the possibility of being used for other purposes, since the nitrosylation effects returned to normal levels within 24 hours (Figure 12). Recovery from drug treatment is important to cell viability and will allow flexibility in the formulation of a final marketable drug treatment.

Protein nitrosylation is a post translational modification. Nitric oxide synthases (NOS) generate nitric oxide, which in turn nitrosylates proteins, peptides and amino acids. The S-nitrosylated proteins are called protein-SNO. These protein-SNO and the direct effects of NO regulate several biological functions such as smooth muscle tone, inflammation, apoptosis and others (Figure 18). The nitroso group from the proteins is transferred to glutathione (GSH) forming S-nitrosoglutathione (GSNO). GSNO can then be converted to GSSG and  $\text{NH}_2\text{OH}$



in the presence of NADH and deplete the GSNO pool. By degradation of GSNO, GSNOR plays an important role in balancing the amount of GSNO and ultimately protein-SNO.

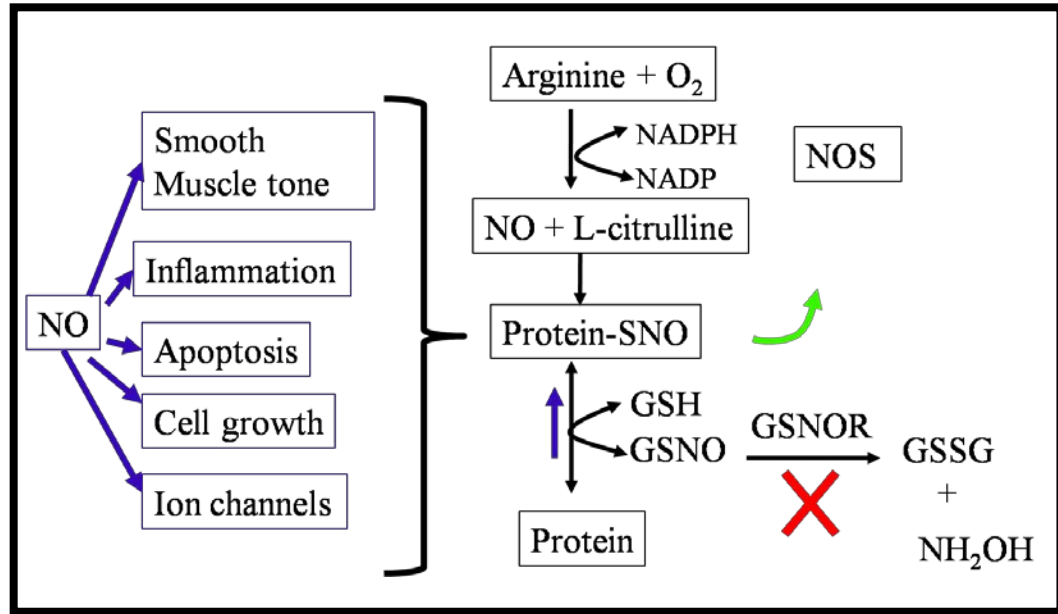


Figure 18. NO Bioactivity and Signaling Pathway. Role of GSNOR inhibition on the NO Signaling Pathway and possibly the bioactivity of NO.

The inhibition of GSNOR will therefore increase the cellular concentration of GSNO and therefore indirectly increases the amount of nitrosylated proteins.

Several GSNOR inhibitors were identified in our laboratory and the aim of this study was to understand their cellular effects. One of the experiments studied the effect of the compound on protein-SNO. As shown in Figure 12, we observed a time dependent increase in protein-SNO. In the same experiment, the treatment with GSNOR inhibitor plus L-NAME inhibited accumulation of nitrosylated

proteins in comparison with the same condition without L-NAME. Since L-NAME is known to inhibit nitric oxide synthases (NOS), this result substantiates that NOS activity is necessary for the observed increase in cellular nitrosylation. This would also indicate that inhibiting GSNOR does not directly increase nitrosylation of proteins but prevents the transfer of NO by decreasing the levels of GSNO.

Next we focused on identification of nitrosylated proteins using protein specific antibodies. As shown in Figure 13 we identified nitrosylation of IKK $\beta$  (Figure 19 Step A). So the question raised was whether nitrosylation of IKK $\beta$  affects its activity. IKK $\beta$  is responsible for phosphorylation of I $\kappa$ B $\alpha$  and phosphorylation of I $\kappa$ B $\alpha$  results in its degradation and activation of NF- $\kappa$ B pathway. Therefore, we studied the phosphorylation of I $\kappa$ B $\alpha$  in the presence of inhibitor C3. After treatment with C3, we expected pI $\kappa$ B levels to be decreased due to nitrosylation of IKK $\beta$ . Instead we discovered pI $\kappa$ B amounts were equivalent in C3 treated and untreated cells. However, we did see an increase in total I $\kappa$ B $\alpha$  (Figure 14). To confirm that the accumulation of I $\kappa$ B was due to decreased phosphorylation and not due to inhibition of proteasomal degradation, cells were treated with inhibitor C3 and MG-132, a known proteasome inhibitor. If the decreased degradation of I $\kappa$ B $\alpha$  was due to inhibition of proteasome then the concentration of C3 should not affect the pI $\kappa$ B $\alpha$  in the presence of MG-132. As shown in Figure 15 we observed a dose-dependent decrease in pI $\kappa$ B. Because of this dose-dependent decrease of pI $\kappa$ B, the direct inhibition of proteasomal

degradation by the compounds is eliminated (Figure 19 Step B). Therefore, inhibition of IKK $\beta$  activity is most likely responsible for the decreased phosphorylation of I $\kappa$ B $\alpha$  (Figure 19 Step C). So the next question was whether the phosphorylation of IKK $\beta$  was affected by nitrosylation (Figure 19 Step D). As seen in Figure 16 we did not detect any change in pIKK $\beta$  with different concentrations of C3 (Figure 1).

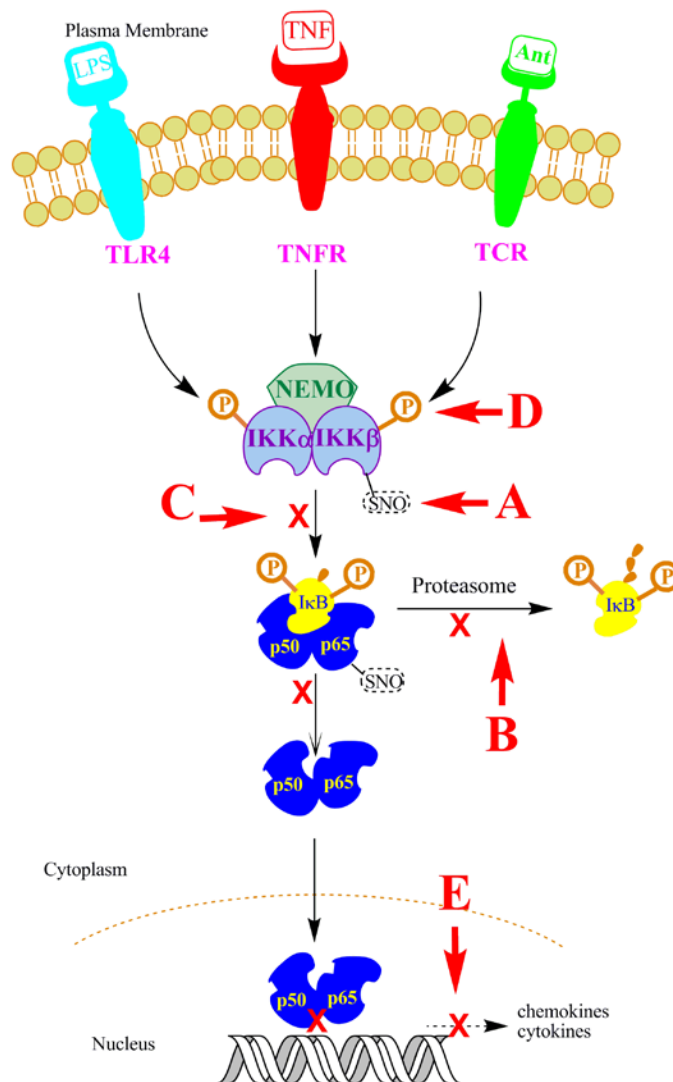


Figure 19. NF- $\kappa$ B Pathway. Effects of inhibiting GSNOR on the NF- $\kappa$ B pathway.

In agreement with our results, inhibition of IKK $\beta$  through S-nitrosylation has been documented (Reynaert *et al.*, 2004). The activity of IKK $\beta$  is inhibited by S-nitrosylation, of the Cys-179 which is between the two phosphorylated serine residues, Ser-177 and Ser-181 (Reynaert *et al.*, 2004). The S-nitrosylation of this cysteine could be hindering the active site of the IKK $\beta$  or affect its structural conformation.

Another potential question would be if IKK $\beta$  activity is inhibited by direct binding of compound to IKK $\beta$ . In a separate experiment done in our lab (data not shown) we showed by an *in vitro* assay that IKK $\beta$  activity could not be inhibited by the compounds.

To determine if the decreased degradation of I $\kappa$ B $\alpha$  caused by C3 translated into decreased NF- $\kappa$ B activity we studied the expression of ICAM, one of the proteins whose expression is regulated by the transcription factor NF- $\kappa$ B. We found a dose-dependent decrease in amounts of ICAM-1 with increasing C3 concentration after 10 hours of exposure (Figure 17). In a separate experiment we also studied the effect of C3 on a stable transfected cell line containing a luciferase gene under control of NF- $\kappa$ B, once again we found a dose-dependent decrease in luciferase activity with increasing concentration of C3 (data not shown). Together this data supports the premise that the activity of transcription factor NF- $\kappa$ B is suppressed by inhibiting GSNOR with compound C3 (Figure 19 Step E).

## II. Biotechnology

During the experimental procedures, several biotechniques were utilized, Western blots, cell culture, ELISA, and gel filtration. Western blots were a major module for detecting changes in protein expression as a result of treatment with GSNOR inhibitors. Choice of antibodies is critical in Western blots. Antibodies can vary from company to company, and lot to lot. We found that NF- $\kappa$ B pathway proteins antibodies from Cell Signaling Technology were more reliable than other sources. Western blots can be very selective, *ie.* monoclonal antibodies, for the detection of proteins as well as extremely sensitive to the amount of the protein. Monoclonal antibodies are specific for the chosen protein using a unique peptide segment. The monoclonal antibody will only bind to a selected peptide and therefore only one antibody binds to one molecule of protein. This will weaken the signal of the antibody and possibly miss a low abundance protein. Polyclonal antibodies can bind to several different peptides on a protein and therefore provide a larger signal. Polyclonal antibodies might bind other nonspecific proteins. The dilution of the antibody was also critical. If the antibody concentration was too low, the signal would be very low and difficult to obtain. If the concentration of the antibody was too high, the blots would show a lot of nonspecific signals which could interfere with the specific protein you were trying to detect. The protein transfer electrophoresis was also very critical in Western blots. The allotted time for transferring was important. If the transfer was not complete, proteins were left behind in the gel. If the transfer was too long the protein will go through the

membrane and onto the filters. Western blots have more specificity in selecting multiple targets than an ELISA or a UV/Vis spectrophotometry protein assay.

Cytotoxicity testing with both Promega One Assay and the BrdU Assay was important. Both assays are based on different principles.

The data analysis for quantitation using ELISAs are less variable. The issue with ELISAs were that they are time consuming and require developing the conditions for the experiment, selection of antibody, its concentration, standard curve, etc.

Protein Assays can be critical to the analysis of data. Several techniques, such as Western blots, are dependent on the amount of protein loaded onto the gel. Some buffers and detergents used to prepare samples can interfere with the protein assay, *ie.* Bradford protein assay is much more sensitive to the amount of Sodium dodecyl sulfate (SDS) in the sample being analyzed compared to BCA assay where SDS does not interfere with the results.

Cell culture techniques played a major role in obtaining data for examining various hypotheses. Sterility in cell culture is critical since contamination such as bacteria, mycoplasma and other cell lines, invalidate the results. Cell culture is used as a middle step between *in vitro* and whole animal studies. Cell culture will demonstrate the potential results under endogenous conditions compared to *in vitro* results. Cell culture is used for testing several different conditions quickly. Cell culture is much cheaper and humane than whole animal studies. Counting and plating cells is critical for consistency within an

experiment as well as reproducibility between experiments. In cell culture, specific functioning cells can be tested *ie.* hepatocytes of the liver to epithelial cells from the lungs. In our studies two cells lines were chosen, mouse Abelson murine leukemia virus transformed macrophage cells, RAW 264.7 and human lung carcinoma epithelial cell line, A549. The RAW 264.7 cells are known as the cytokine-treated murine macrophage which has been established as a prototype for studying NO-related metabolic pathways for nitrosative stress in mammalian cells (Eu *et al.*, 2000).

## CONCLUSION

Activation of NF- $\kappa$ B pathway by TNF $\alpha$  is a well defined: IKK $\beta$  activation by phosphorylation which then phosphorylates I $\kappa$ B, ubiquination and consequently degradation of pI $\kappa$ B from the cytosolic I $\kappa$ B-NF- $\kappa$ B complex by Proteasome 26. This degradation of I $\kappa$ B exposes the nuclear translocation domain allowing NF- $\kappa$ B to enter the nucleus. Once in the nucleus, NF- $\kappa$ B is the transcription factor and main regulator of the immune response proteins, cytokines and chemokines (Janssen-Heininger *et al.*, 2009).

The research provided above supports the suggested hypothesis that inhibiting GSNOR by C3 will suppress the activation of the transcription factor NF- $\kappa$ B indirectly through the regulation of S-nitrosylation of IKK $\beta$ .

### *Future Therapy for Asthma Patients*

GSNOR activity is specifically inhibited by C2 and C3. Given that GSNOR has been declared an “important regulator of Human Asthma” (Que *et al.*, 2009) managing the activity of this enzyme through inhibition could be a major advancement in Human Asthma relief and other lung diseases (Staab *et al.*, 2009) Perhaps with key formulation design of the inhibitor, GSNOR can be regulated using C2 or C3 as a future treatment for asthma.



## REFERENCES

- Barber,R.D. and Donohue,T.J. (1998). Pathways for transcriptional activation of a glutathione-dependent formaldehyde dehydrogenase gene. *J. Mol. Biol.* *280*, 775-784.
- Bernas,T. and Dobrucki,J. (2002). Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. *Cytometry.* *47*, 236-242.
- Bove,P.F. and van der Vliet,A. (2006). Nitric oxide and reactive nitrogen species in airway epithelial signaling and inflammation. *Free Radic. Biol. Med.* *41*, 515-527.
- Estonius,M., Svensson,S., and Hoog,J.O. (1996). Alcohol dehydrogenase in human tissues: localisation of transcripts coding for five classes of the enzyme. *FEBS Lett.* *397*, 338-342.
- Eu,J.P., Liu,L., Zeng,M., and Stamler,J.S. (2000). An apoptotic model for nitrosative stress. *Biochemistry.* *39*, 1040-1047.
- Foster,M.W., McMahon,T.J., and Stamler,J.S. (2003). S-nitrosylation in health and disease. *Trends Mol. Med.* *9*, 160-168.
- Fukuto,J.M., Bartberger,M.D., Dutton,A.S., Paolocci,N., Wink,D.A., and Houk,K.N. (2005). The physiological chemistry and biological activity of nitroxyl (HNO): the neglected, misunderstood, and enigmatic nitrogen oxide. *Chem. Res. Toxicol.* *18*, 790-801.
- Gaston,B., Reilly,J., Drazen,J.M., Fackler,J., Ramdev,P., Arnette,D., Mullins,M.E., Sugarbaker,D.J., Chee,C., Singel,D.J., and . (1993). Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. *Proc. Natl. Acad. Sci. U. S. A.* *90*, 10957-10961.

Hayden,M.S., West,A.P., and Ghosh,S. (2006). NF-kappaB and the immune response. *Oncogene*. 25, 6758-6780.

Holmquist,B. and Vallee,B.L. (1991). Human liver class III alcohol and glutathione dependent formaldehyde dehydrogenase are the same enzyme. *Biochem. Biophys. Res. Commun*. 178, 1371-1377.

Hur,M.W. and Edenberg,H.J. (1992). Cloning and characterization of the ADH5 gene encoding human alcohol dehydrogenase 5, formaldehyde dehydrogenase. *Gene*. 121, 305-311.

Jaffrey,S.R. and Snyder,S.H. (2001). The biotin switch method for the detection of S-nitrosylated proteins. *Sci. STKE*. 2001, L1.

Janssen-Heininger,Y.M., Poynter,M.E., Aesif,S.W., Pantano,C., Ather,J.L., Reynaert,N.L., Ckless,K., Anathy,V., van der Velden,J., Irvin,C.G., and van der Vliet,A. (2009). Nuclear factor kappaB, airway epithelium, and asthma: avenues for redox control. *Proc. Am. Thorac. Soc*. 6, 249-255.

Kaiser,R., Holmquist,B., Vallee,B.L., and Jornvall,H. (1989). Characteristics of mammalian class III alcohol dehydrogenases, an enzyme less variable than the traditional liver enzyme of class I. *Biochemistry*. 28, 8432-8438.

Liu,L., Hausladen,A., Zeng,M., Que,L., Heitman,J., and Stamler,J.S. (2001). A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature*. 410, 490-494.

Liu,L., Yan,Y., Zeng,M., Zhang,J., Hanes,M.A., Ahearn,G., McMahon,T.J., Dickfeld,T., Marshall,H.E., Que,L.G., and Stamler,J.S. (2004). Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell*. 116, 617-628.

Que,L.G., Liu,L., Yan,Y., Whitehead,G.S., Gavett,S.H., Schwartz,D.A., and Stamler,J.S. (2005). Protection from experimental asthma by an endogenous bronchodilator. *Science* 308, 1560-1561.

- Que,L.G., Yang,Z., Stamler,J.S., Lugogo,N.L., and Kraft,M. (2009). S-Nitrosogluthathione Reductase -- An Important Regulator in Human Asthma. *Am. J. Respir. Crit Care Med.* *April 24*.
- Reynaert,N.L., Ckless,K., Korn,S.H., Vos,N., Guala,A.S., Wouters,E.F., van der Vliet,A., and Janssen-Heininger,Y.M. (2004). Nitric oxide represses inhibitory kappaB kinase through S-nitrosylation. *Proc. Natl. Acad. Sci. U. S. A.* *101*, 8945-8950.
- Sanghani,P.C., Davis,W.I., Fears,S.L., Green,S.L., Zhai,L., Tang,Y., Martin,E., Bryan,N.S., and Sanghani,S.P. (2009). Kinetic and cellular characterization of novel inhibitors of S-nitrosogluthathione reductase. *J. Biol. Chem.*
- Sanghani,P.C., Stone,C.L., Ray,B.D., Pindel,E.V., Hurley,T.D., and Bosron,W.F. (2000). Kinetic mechanism of human glutathione-dependent formaldehyde dehydrogenase. *Biochemistry.* *39*, 10720-10729.
- Staab,C.A., Hellgren,M., Grafstrom,R.C., and Hoog,J.O. (2009). Medium-chain fatty acids and glutathione derivatives as inhibitors of S-nitrosogluthathione reduction mediated by alcohol dehydrogenase 3. *Chem. Biol. Interact.* *180*, 113-118.
- Staab,C.A., Hellgren,M., and Hoog,J.O. (2008). Medium- and short-chain dehydrogenase/reductase gene and protein families : Dual functions of alcohol dehydrogenase 3: implications with focus on formaldehyde dehydrogenase and S-nitrosogluthathione reductase activities. *Cell Mol. Life Sci.* *65*, 3950-3960.
- Wagner,F.W., Pares,X., Holmquist,B., and Vallee,B.L. (1984). Physical and enzymatic properties of a class III isozyme of human liver alcohol dehydrogenase: chi-ADH. *Biochemistry.* *23*, 2193-2199.
- Wang,X., Kettenhofen,N.J., Shiva,S., Hogg,N., and Gladwin,M.T. (2008). Copper dependence of the biotin switch assay: modified assay for measuring cellular and blood nitrosated proteins. *Free Radic. Biol. Med.* *44*, 1362-1372.
- Whiteman,S.C. and Spiteri,M.A. (2008). IFN-gamma regulation of ICAM-1 receptors in bronchial epithelial cells: soluble ICAM-1 release inhibits human rhinovirus infection. *J. Inflamm. (Lond).* *5*, 8.

Yang,Z.N., Bosron,W.F., and Hurley,T.D. (1997). Structure of human chi chi alcohol dehydrogenase: a glutathione-dependent formaldehyde dehydrogenase. *J. Mol. Biol.* 265, 330-343.

Zaman,K., Hanigan,M.H., Smith,A., Vaughan,J., Macdonald,T., Jones,D.R., Hunt,J.F., and Gaston,B. (2006). Endogenous S-nitrosoglutathione modifies 5-lipoxygenase expression in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 34, 387-393.

Zhang,Y., Keszler,A., Broniowska,K.A., and Hogg,N. (2005). Characterization and application of the biotin-switch assay for the identification of S-nitrosated proteins. *Free Radic. Biol. Med.* 38, 874-881.

# **CURRICULUM VITAE**

**Sharry L. Fears**

## **EDUCATION**

Masters of Science in Biochemistry and Molecular Biology, 2009,  
Indiana University

Graduate Certificate of Biotechnology, 2006, Indiana University  
Department of Biochemistry and Molecular Biology

Bachelor of Science in Biochemistry, 1983, Purdue University

## **SHORT COURSES**

AMA: Management Skills and Techniques for New First-Line Supervisors

ACS Short Course: Fundamentals of Experimental Design

The Center for Professional Advancement: Drug Product Stability and  
Shelf-Life

## **PUBLICATIONS**

Sanghani,P.C., Davis,W.I., Fears,S.L., Green,S.L., Zhai,L., Tang,Y.,  
Martin,E., Bryan,N.S., and Sanghani,S.P. (2009). *Kinetic and cellular  
characterization of novel inhibitors of S-nitrosoglutathione reductase. J.  
Biol. Chem.*

Mello T, Nakatsuka A, Fears S, Davis W, Tsukamoto H, Bosron WF,  
Sanghani SP (2008) *Expression of carboxylesterase and lipase genes in  
rat liver cell-types.Biochem Biophys Res Commun.* 374, 460-4.

## EXPERIENCE

2005 – Current

**Research Associate, Indiana University School of Medicine,  
Department of Biochemistry and Molecular Biology**

**Masters Research:** *Effect of Inhibition of S-nitrosoglutathione Reductase on the NF- $\kappa$ B Pathway.* Investigated the effects of small molecules on the NF- $\kappa$ B signaling pathway, for potential cancer or anti-inflammatory drug; Working with two cells lines, RAW 264.7 murine macrophage cells and A549 lung epithelial cells, studying cell proliferation, apoptosis, nitrosylation of proteins, and effects on inflammatory signaling pathways

**Research:** Kinetics on a cancer drug with enzymes, developed HPLC and Solid Phase Extraction method to quantitate substrate and product; Kinetic Assay of Carboxylesterase with Benazepril using LC/MS; Isolated hepatic stellate cells from rat liver and tested lysates for different lipase enzymes by Western blot, and activity assays (methods used: UV/Vis Spectrophotometry, Fluorescence Spectrophotometry and HPLC)

**Experience in the following Techniques:** Protein Expression & Purification in E.coli, Electrophoresis, Western blot, maintenance of several cell lines, Cytotoxicity, ELISA, Immunoprecipitation, Immunostaining, Fluorescence Microscopy, DNA and RNA isolation, Plasmid Purification, Transfections, PCR, and quantitative PCR, HPLC, Solid Phase Extraction, Kinetics, Maldi Mass Spectroscopy, LC/MS

**Manage lab:** Purchasing equipment and consumables, maintenance and scheduling of equipment; Write and test Protocols, and prepare all materials for the graduate lab classes, Proteomics and Cell Biology, and then assist students during the laboratory class

**1983 - 1992**

**Pitman-Moore Research and Development Pharmaceutical Division  
1989 Associate Research Scientist**

Directed all NADA stability studies; Designed experiments to obtain data for FDA Submissions; Received and analyzed stability data following GLP; Communicated stability experiment results to product team; Authored NADA stability sections

Discovered a stability problem, too much water in a protein formulation, and helped reformulate a more stable product

Discovered a major storage problem in the diluent of a key product; Developed new product formulations; Supervised full and part time lab technicians

**1987 Assistant Research Scientist**

Monitored and coordinated NADA stability studies; Maintained database for stability studies; Preformulated and formulated new products; Supervised full-time lab technician

**1983 Research Technician**

Developed and evaluated protein formulations

Tested formulations using Protein Analysis of protein concentrations by Bio Rad Protein Assay, UV Spectrum, and SDS-PAGE Gels; Lyophilization of protein formulations

Liaison between Formulation Department and Animal Scientists during *in vivo* testing of delivery systems, participated in start up of animal studies and recovery of implants

Maintained animal testing database; Participated in new product evaluation